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Get out or die trying: Peptide- and protein-based endosomal escape of RNA therapeutics

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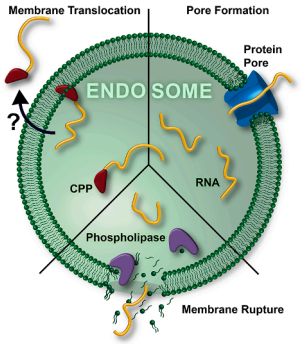
HIGHLIGHTS

- Cell penetrating peptides may enhance cellular uptake but not endosomal escape.
- Proteins can efficiently enhance endosomal escape and enable gene delivery.
- Protein-based endosomal escape is mediated by pore formation or membrane lysis.

GRAPHICAL ABSTRACT



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ABSTRACT

RNA therapeutics offer great potential to transform the biomedical landscape, encompassing the treatment of hereditary conditions and the development of better vaccines. However, the delivery of RNAs into the cell is hampered, among others, by poor endosomal escape. This major hurdle is often tackled using special lipids, polymers, or protein-based delivery vectors. In this review, we will focus on the most prominent peptide- and protein-based endosomal escape strategies with focus on RNA drugs. We discuss cell penetrating peptides, which are still incorporated into novel transfection systems today to promote endosomal escape. However, direct evidence for enhanced endosomal escape by the action of such peptides is missing and their transfection efficiency, even in permissive cell culture conditions, is rather low. Endosomal escape by the help of pore forming proteins or phospholipases, on the other hand, allowed to generate more efficient transfection systems. These are, however, often hampered by considerable toxicity and immunogenicity. We conclude that the perfect enhancer of endosomal escape has yet to be devised. To increase the chances of success, any new transfection system should be tested under relevant conditions and guided by assays that allow direct quantification of endosomal escape.

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1. Introduction

1.1. RNA-based therapies

RNA drugs hold immense potential for advancing both biochemical and medical research, particularly in the realm of personalized treatments. There are different ways how RNA-based therapeutics exert their function, including transient expression of a therapeutic protein achieved by the transfection of messenger RNA (mRNA) as well as modulating endogenous gene expression by RNA interference (RNAi), antisense oligonucleotides (ASOs) or RNA activation (RNAa) [1,2].

Transfection of cells with synthetic mRNA resulting in the expression of a protein of interest is, for example, the basis of mRNA vaccines [3], and as such it has recently gained great importance in fighting the SARS-CoV-2 pandemic [4]. However, mRNA molecules are considerably large. The open reading frame of the SARS-CoV-2 spike protein, for example, counts 1273 amino acids (uniprot ID P0DTC2). Reverse translated to RNA, this constitutes 3819 base pairs (bp). If regulatory sequences are added, then this molecule will count more than 4000 bp, corresponding to a molecular weight in the range of 1280 kDa. According to the measurements of Roth *et al.* for the dimensions of single stranded DNA [5], this translates to a molecule with a length of ca. 2.7 μm in an uncomplexed state. Additionally, native mRNA, like any other nucleic acid, is highly negatively charged, which also contributes to preventing its passive permeation through biological membranes. While these properties make the delivery of mRNA challenging, it has advantages over DNA. The mRNA only has to reach the cytoplasm, where it can be readily translated without having to enter the nucleus [6]. Moreover, only small amounts of an mRNA molecule must arrive in the cytoplasm in order to express a significant number of proteins, considering that many endogenous genes in a cell are expressed with single-digit copies of mRNA molecules [7].

Another prominent RNA-based therapy is based on RNAi, which is an endogenous cellular defense mechanism that can be used as powerful tool to specifically reduce the expression of distinct genes. This is performed by different classes of RNAs, such as small interfering RNAs (siRNA), micro RNAs (miRNA), and small hairpin RNAs (shRNA). Like mRNA, these various classes of RNA readily exert their function in the cytoplasm by forming a complex with endogenous enzymes [8]. The RNA-protein complexes subsequently target mRNAs in a sequence specific manner, which can result in mRNA degradation or translational arrest and, thereby, in reduced protein expression. The RNA molecules used for RNAi are much smaller than mRNA. Generally, they are 15–30 nucleotides long, which corresponds to a molecular weight of ca. 5–10 kDa and, typically, around 2 nm in diameter and 7.5 nm in length [9]. However, the molecules are still too large and hydrophilic to diffuse through the membrane [10]. In the case of siRNA, it was reported in microinjection studies that several hundred copies are required to generate a significant effect within a single cell [11]. Despite this, several therapies based on RNAi have been approved by the FDA, including patisiran, givosiran and lumasiran [12–14].

ASOs are short RNA or DNA oligonucleotides which target specific complementary mRNA sequences in the cytoplasm or the nucleus. After base pairing with those target sequences, ASOs can alter protein expression with the help of distinct enzymes, for example by degradation of target mRNA, regulation of mRNA splicing and inhibition or activation of translation [15]. Therefore, ASOs are a potent tool for the regulation of gene expression, as underlined by the approval of several ASOs-based drugs, such as fomivirsen and casimersen [16,17].

In contrast to RNAi, RNAa is a mechanism to enhance or activate expression of a specific protein. This is achieved with the help of small activating RNAs (saRNA), which form complexes with endogenous enzymes inside the nucleus and bind to specific regulatory regions in the genome controlling a gene of interest. This is followed by the recruitment of specific co-factors resulting in epigenetic and transcriptional alterations and, eventually, in enhanced or activated gene expression [18,19]. Both ASOs and saRNA have molecular properties similar to siRNAs and are, hence, also expected to require rather high copy numbers per cell for exerting their functions efficiently [20,21].

1.2. The main route into cells

RNA molecules, native or chemically modified, are either delivered naked, conjugated to a moiety which helps with cellular uptake and endosomal escape, or complexed inside lipid- or polymer-based nanoparticles [22]. After administration, they are taken up by cells *via* the endocytic pathway. Endocytosis is the process of engulfing extracellular substances with the plasma membrane and subsequent budding off resulting in internalization of the substances in endocytic vesicles. These vesicles, which have a size of 60–120 nm, can fuse with early endosomes (EE), and thereby release their content into the EE lumen. The EE, which have a diameter of 100–500 nm, then typically transform by maturation to recycling endosomes (RE) or to late endosomes (LE). Eventually, the maturation reaches its final stage with the transformation of LE to lysosomes. Importantly, during this maturation process the lumenal pH decreases steadily until reaching an acidic pH of around 4.5–5 in lysosomes. Additionally, lumenal ion concentrations, such as calcium, undergo drastic changes during the maturation from EE to lysosomes [1,23–26]. Moreover, the lysosomal lumen is a very harsh and degradative environment containing various hydrolytic enzymes, such as nucleases, proteases and lipases. These degradative enzymes ensure that cargo arriving in the lysosomes is quickly degraded [27]. Consequently, it is crucial for the success of RNA-based therapeutics to escape the endosomal compartment before reaching the lysosomes. A second reason, why endosomal escape should happen before lysosomal maturation is the observation that lysosomal damage or even rupture is a common danger signal in cells and known to induce apoptosis [28]. Unfortunately, most RNA-based therapeutics are eventually degraded inside lysosomes, making endosomal escape one of the major challenges for the cytoplasmic delivery of RNA as well as other macromolecules [10,29,30].

How can the endosomal membrane be breached? Evolution over millions of years resulted in distinct ways to escape endosomes, as manifested by many pathogens, such as viruses [31–34]. Enveloped viruses, for example, acquired the elegant ability to fuse their membrane with the endosomal limiting membrane. This results in sustained endosomal integrity while releasing viral content into the cytoplasm [32]. Unenveloped entities, on the other hand, evolved distinct ways to evade the endosomal compartment. The general strategy is to rupture endosomes enzymatically resulting in release of the pathogens to the cytoplasm [31,34]. This is, for example, performed by pore-forming proteins, phospholipid degrading enzymes or combinations thereof [31–34].

Here, we review the current progress made on endosomal escape of nucleic acid-based drugs, with a particular focus on RNA therapeutics (although some examples refer to DNA-based systems), mediated by peptide- and protein-based endosomal escape enhancers. This involves the prominent and widely used cell penetrating peptides (CPPs), as well as protein-based enhancers, such as phospholipases and pore-forming proteins. Other endosomal escape strategies, including lipoplex- and polyplex-mediated endosomal escape, will not be addressed in this manuscript and the reader is referred to other recent review articles for more information on these topics [35–37].

2. Peptides for endosomal escape

CPPs, so-called due to their alleged ability to penetrate the endosomal or plasma membranes, are one of the most studied non-viral RNA delivery vectors. The peptides are usually positively charged and readily form nanoparticles when mixed with RNA molecules [38]. In some applications the cationic peptides were also covalently conjugated to the RNA [39]. In cell culture experiments, it was shown that CPPs are able to

achieve cytoplasmic delivery of various cargos [40]. However, evidence is amassing that CPPs are not promoting membrane translocation and, hence, endosomal escape and that their beneficial effects on transfection are likely originating from increased cell surface binding and uptake under specific cell culture conditions, such as low serum concentrations. Here, we will discuss how CPPs are used in nucleic acid transfection *in vitro* and *in vivo*, and how the endosomal escape efficiency of these entities was quantified. The putative mechanisms how CPPs penetrate membranes have been summarized in many review articles and will, therefore, not be reviewed here [30,38–42].

2.1. Cell penetrating peptides applied in vitro

Muratovska *et al.* were one of the first groups to use CPPs in 2004 for the delivery of siRNA into mammalian cells [43]. In our view, this study is an example for the development and assessment of peptide-based endosomal escape enhancers. The investigators used a set of techniques and protocols that are applied to the present day. While this early study was performed thoroughly and its results were communicated in a scientifically accurate manner, we believe that the applied methods, *e.g.* low serum concentrations, long transfection times and high RNA concentrations, can easily lead to misinterpretation and overestimation of the efficacy of the transfection system. We, therefore, discuss the study here in some detail.

The authors tested the peptides penetratin and transportan (Table 1), which were coupled to the siRNA molecules via disulfide bridges. The *in vitro* transfection efficiency was determined on different cell lines using 25 nM CPP-siRNA conjugates in 0–10 % serum and transfection times of up to seven days. The authors assessed the transfection efficiency by fluorescence microscopy analysis and flow cytometry, evaluating the decrease in luciferase or green fluorescent protein (GFP) signal. They demonstrated that penetratin as well as transportan increased transfection of siRNA compared to Lipofectamine in a cell culture setting under non-challenging conditions.

In a typical cell culture setting, as the one applied in the study by Muratovska *et al.* [43], 25 nM siRNA would correspond to around 45 million siRNA molecules per cell (assuming 100′000 cells per well in 300 μL medium). Considering that as little as a few hundred siRNA copies are required in the cytoplasm to have a significant impact on target protein downregulation [11], the applied dose was indeed high. This indicates that only a very small fraction of the siRNA reached the cytoplasm, while most of the payload must have remained in either the culture medium, on the cell surface, the endolysosomal compartment, or entered the cytoplasm in a non-bioavailable fashion, *e.g.* partially degraded. Interestingly, other studies [44–48] demonstrated that the highly positive charge of CPPs, such as the often-used TAT, resulted in an increased cellular association and uptake of the conjugated cargo in low serum concentrations. This is expected since the cell surface is typically negatively charged [49]. In the absence of serum proteins or with just 10 % serum, the cationic peptides are likely to retain their positive zeta potential and interact strongly with the cell surface. In full serum, the cationic charges of the peptides would be more efficiently shielded by serum components, and the charge-based cell association as well as cell uptake would be mitigated. Serum-dependent alterations of particle surface charge was shown for several systems including poly (ethylenimine) (PEI) nanoparticles [50]. Additionally, increasing serum concentrations were shown to proportionally decrease the transfection efficiency of a CPP-based system, as demonstrated by Morais *et al.* testing a CPP-based siRNA delivery system in up to 60 % serum [51]. This indicates that the bottleneck of peptide-based RNA delivery remains the penetration of the cellular membranes, *i.e.* the plasma membrane or endosomal membrane.

In the past 19 years, many novel CPPs were developed to improve the efficiency of peptide-based RNA delivery. The various peptide systems were frequently summarized, *e.g.* recently by Falato *et al.* [52] as well as by Yokoo *et al.* [53], and are therefore not discussed here in detail. Shortly, CPPs used to deliver siRNA include variations of the PepFect [54–58], NickFect [56,59,60], and CADY [61,62] systems, which are complexing siRNA; as well as *e.g.* LMWP [63] and CKRRMKWKK [64], which have been used as conjugates to siRNA. CPPs developed for the delivery of mRNA include TAT [65], GALA [66] and PepFect [67]. Transfection agents for miRNA include PepFect and CADY [58] as well as LK [68]. Table 1 provides an overview of the various systems applied *in vitro* with emphasis on the transfection conditions. The table indicates the lowest tested RNA concentrations that resulted in a significant effect as well as the percentage of serum in the transfection medium. Importantly, it only shows a selection of key publications on this topic and is not intended to be exhaustive. The included studies were selected based on the applied CPP and on the availability of sufficiently detailed transfection protocols. Generally, the systems were developed and tested in serum concentrations ranging from 0 to 10 %, with very few studies assessing transfection data in up to 60 % serum [51]. Biologically relevant serum concentrations, *i.e.* above 90 %, were, to the best of our knowledge, not tested.

We investigated how the transfection efficiencies of peptide-based RNA delivery systems have evolved since the early studies outlined above. Unfortunately, most studies do not provide an EC50 value (*i.e.* the siRNA concentration required to reach a half maximal knockdown effect) for their respective CPP system and the studies were performed on various cell lines under strongly different conditions. Therefore, we analyzed the cell culture studies in Table 1, and plotted the lowest tested RNA concentrations that resulted in a significant cellular effect (*e.g.* target protein knockdown for siRNA) against the respective year of publication (Fig. 1). This provides a rough estimate on the change in transfection efficiencies in the field. Importantly, the administered RNA dose did not decrease over time and remained in the high nM range. While novel CPP-based systems are emerging, there is no evidence for an increase in transfection efficiency *in vitro*.

2.2. Endosomal escape efficiency

In order to determine if CPPs are indeed promoting endosomal escape, the efficiency of the latter must be measured. However, the direct quantification of peptide-based endosomal escape of nucleic acids is challenging, and reliable assays are currently missing. As discussed above, the tracking of labelled nucleic acids can lead to artifacts, due to the limited distinguishability of the different RNA populations that are either bound to the cell surface, entrapped in endosomes or dispersed in the cytoplasm. On the other hand, measuring the effect exerted by a successfully transfected RNA inside the cell only provides an indirect quantification of the endosomal escape efficiency. However, direct quantification assays do exist for peptide mediated endosomal escape of protein cargo, and they provide interesting insights into the strengths and weaknesses of CPPs.

Lundberg *et al.* demonstrated in the year 2003 that various CPPs, including TAT, fused to GFP, were indeed increasing cell surface binding and uptake in low serum concentrations [96]. However, they noticed that the CPPs did not seem to increase endosomal escape of the fluorescent cargo and that previous observations of successful cytoplasmic delivery were indeed misinterpretations of artifacts occurring from cell fixation. A more elaborate study by Teo *et al.* recently confirmed these results [97]. They developed an elegant assay to directly quantify the efficiency of endosomal escape. In a method called Split Luciferase Endosomal Escape Quantification (SLEEQ), the high affinity complementary peptide (HiBiT), which is part of split luciferase, was fused to GFP as a model cargo protein and the system was incubated with HeLa cells (Fig. 2). The cells, on the other hand, were stably expressing an actin-bound large BiT protein (LgBiT), which comprises the other part of the split luciferase. This LgBit domain is, hence, sequestered to the actin filaments in the cytoplasm of cells. LgBiT alone is inactive and cannot produce a bioluminescent signal, until it is complemented with the HiBiT counterpart. Therefore, bioluminescence is only detected in the

Table 1
Summary of key studies using CPP-based systems for the delivery of RNA *in vitro*.

CPP	Sequence	Cargo coupling	Cargo	Cargo concentration (nM)	FBS concentration (%)	Cell line	Year	Ref.
Penetratin	CRQIKIWFQNRRMKWKK	Conjugate	siRNA	25	n.s.	COS-7, C166, EOMA	2004	[43]
Transportan	CLIKKALAALAKLNIKLLYGASNLTWG	Conjugate	siRNA	25	n.s.	COS-7, C166, EOMA	2004	[43]
Cholesteryl oligo-d-arginine	Cholesteryl-RRRRRRRRR	Complex	siRNA	~47*	0	CT-26	2006	[69]
EB1	LIRLWSHLIHIWFQNRRLKWKKK	Complex	siRNA	10	0, 10	HeLa, HepG2	2007	[70]
Bovine Prp (1–30)	MVKSKIGSWILVLFVAMWSDVGLCKKRPKP	Complex	siRNA	50	0	HeLa, HepG2	2007	[70]
MPGΔ^{NLS}	GALFLGWLGAAGSTMGAPKSKRKV	Complex	siRNA	10	0	HeLa, HepG2	2007	[70]
Stearyl-R_8	Stearyl-RRRRRRRR	Complex	siRNA	60	0	HeLa	2007	[71]
TP10	AGYLLGKINLKALAALAKKIL	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
Stearyl-TP10	Stearyl-AGYLLGKINLKALAALAKKIL	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
R_9	RRRRRRRRR	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
Stearyl-R_9	Stearyl-RRRRRRRRR	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
Penetratin	RQIKIWFQNRRMKWKK	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
Stearyl-Penetratin	Stearyl-RQIKIWFQNRRMKWKK	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Complex	2′-OMe RNA	200	0	HeLa	2008	[72]
TAT-U1A RNA binding domain	GRKKRRQRRRPPQC-U1A	Complex	siRNA	200	0	CHO	2008	[73]
PTD-dsRNA binding domain (dsRBD)	MGRKKRRQRRRGHSGRKKRRQRRRGHIYPYDVPDYAGDPGRKKRRQRRR-dsRBD	Complex	siRNA	100	0	H1299, HaCat, HFF, B16F0, T98G, HUVEC, Jurkat T, THP-1	2009	[74]
CADY	Acetyl-GLWRALWRLLRSLWRLLWRA-cysteamide	Complex	siRNA	0.6	0	U_2OS, THP1, 3T3C, HUVEC	2009	[61]
Stearoyl peptide	Stearyl-CHHRRRRHHC	Complex	siRNA	~77*	0	S-180	2010	[75]
Stearoyl peptide	Stearyl-GHHRRRRHHG	Complex	siRNA	~77*	0	S-180	2010	[75]
PepFect6	Stearyl-AGYLLGK(K(K$_2$(trifluoromethylquinoline$_4$)))INLKALAALAKKIL[1]	Complex	siRNA	6	0, 10	$U_2$0S, HEK293, HeLa, CHO, RD4, Hepa1c1c7, Bhk21, HepG2, primary MEF, Jurkat	2011	[54]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	siRNA,	50	n.s.	HeLa	2011	[55]
PLL-CA	Poly(L-lysine-cholic acid)	Complex	siRNA	50	10	PC3, TRAMP C1,	2012	[76]
NickFect	Stearyl-TP10 analogs[1]	Complex	siRNA	25	0, 10	CHO	2013	[59]
Myr-TP-Transferrin targeting peptide (Tf)	Myristyl-GWTLNSAGYLLGKINLKALAALAKKIL-Tf	Complex	siRNA	~40*	0	U87	2014	[77]
Hph1-Hph1-dsRBD	YARVRRRGPRRGHYARVRRRGPRR-dsRBD	Complex	siRNA	100	0	HeLa	2014	[78]
TAT-TAT-dsRBD	RKKRRQRRRGHYPYDVPDYAGDRKKRRQRRR-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
TAT-TAT-TAT-dsRBD	RKKRRQRRRGHYPYDVPDYAGDRKKRRQRRRGDPAGSRKKRRQRRRR-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
PTD4-PTD4-dsRBD	YARAAARQARARSYARAAARQARALQYPYDVPDYA-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
PTD4-PTD4-MPG-MPG	YARAAARQARARSYARAAARQAR-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
Hph1-Hph1-dsRBD	YARVRRRGPRRGHYARVRRRGPRRRR-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
Hph1-Hph1-dsRBD	YARVRRRGPRRGHYARVRRRGPRRRR-dsRBD	Complex	siRNA	50	0	HeLa	2014	[79]
CA-R_8	Capryl-RRRRRRRR	Complex	siRNA	100	0	HepG2, A549	2015	[80]
StA-R_8	Stearyl-RRRRRRRR	Complex	siRNA	100	0	HepG2, A549	2015	[80]
OA-R_8	Oleyl-RRRRRRRR	Complex	siRNA	100	0	HepG2, A549	2015	[80]
LA-R_8	Linolyl-RRRRRRRR	Complex	siRNA	100	0	HepG2, A549	2015	[80]
RGD10-10R	DGARYCRGDCFDGRRRRRRRRRR	Complex	siRNA	80	0	MDA-MB-231	2015	[81]
PepFect6	Stearyl-AGYLLGK(K(K$_2$(trifluoromethylquinoline$_4$)))INLKALAALAKKIL[1]	Complex	miRNA	30	0	Human primary keratinocytes	2016	[58]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	miRNA	30	0	Human primary keratinocytes	2016	[58]

(*continued on next page*)

Table 1 (*continued*)

CPP	Sequence	Cargo coupling	Cargo	Cargo concentration (nM)	FBS concentration (%)	Cell line	Year	Ref.
CADY	GLWRALWRLLRSLWRLLWRA	Complex	miRNA	30	0	Human primary keratinocytes	2016	[58]
CADY	GLWRALWRLLRSLWRLLWKA-cysteamide	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
CADY-K	GLWRALWRLLRSLWRLLWK	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
S-CADY	GWRALWRLWRSLWRA	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
CADY-H	GLWHALWHLLHSLWHLLWHA	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
PSW	GLWRALWRLWRSLWRLLWKA	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
PSR	GLWRALWRLLRSLWRLWRKA	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
PG09	GLWRALWRALWRSLWRLKRKV	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
PG16	GLWRALWRGLRSLWRLLWKV	Complex	siRNA	20	0	Neuro-2A, B16-F10	2016	[62]
STR-KV	Stearyl-HHHKKKVVVVVV	Complex	siRNA	50	0	A549, CHO, HeLa,	2016	[82]
CKRRMKWKK	CKRRMKWKK	Conjugate	siRNA	100	0	HT-1080	2016	[64]
LMWP-PEG	VSRRRRGGRRRRRR-PEG	Conjugate	siRNA	50	0	MDA-MB-231	2017	[63]
NickFect51	Stearyl-AGYLLGδOINLKALAALAKKIL	Complex	siRNA	25	10	U87	2017	[56]
NickFect57	Stearyl-AGYLLGδOINLKALAALAKAIL	Complex	siRNA	25	10	U87	2017	[56]
PepFect3	Stearyl-AGYLLGKINLKALAALAKKIL	Complex	siRNA	25	10	U87	2017	[56]
TP10	AGYLLGKINLKALAALAKKIL	Complex	siRNA	25	10	U87	2017	[56]
PepFect6	Stearyl-AGYLLGK(K(K$_2$(trifluoromethylquinoline$_4$)))INLKALAALAKKIL[1]	Complex	siRNA	25	10	U87	2017	[56]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	siRNA	25	10	U87	2017	[56]
PL$_9$R	1-myristoyl-2-(14-carboxymyristoyl)-*sn*-glycero-3-phosphocholine-RRRRRRRRR	Complex	siRNA	1000	10	HeLa	2017	[83]
RICK (*retro*-inverso form of CADY-K)	kwllrwlsrllrwlarwlg	Complex	siRNA	5	0	U87	2017	[84]
CADY-K	GLWRALWRLLRSLWRLLWK	Complex	siRNA	5	0	U87	2017	[84]
D-Cady-K	glwralwrllrslwrllwk	Complex	siRNA	5	0	U87	2017	[84]
PEG-RICK	PEG-kwllrwlsrllrwlarwlg	Complex	siRNA	5	0	U87	2017	[85]
RALA	WEARLARALARALARHLARALARALRACEA	Complex	mRNA	-147*	0	DC2.4	2017	[86]
gH625	HGLASTLTRWAHYNALIRAFGGG	Complex	siRNA	50	0	MDA-MB-231	2018	[87]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	siRNA	10	0	hES H9, H1	2019	[57]
gH625	HGLASTLTRWAHYNALIRAFGGG	Complex	siRNA	50	0	MDA-MB-231	2019	[88]
NickFect71	Stearyl-HHYHHGOδILLKALKALAKAIL	Complex	siRNA	25	10	CHO, U87	2019	[60]
NickFect700	HHHHYHHGOδILLKALKALAKAIL	Complex	siRNA	25	10	CHO, U87	2019	[60]
NickFect704	Stearyl-HHHHHHGOδILLKALKALAKAIL	Complex	siRNA	25	10	CHO, U87	2019	[60]
NickFect707	Stearyl-HHHHHHYLLGOδILLKALKALAKAIL	Complex	siRNA	25	10	CHO, U87	2019	[60]
NickFect721	Stearyl-HHHHHHYHHGOδILLKALKALAKAIL	Complex	siRNA	25	10	CHO, U87	2019	[60]
KL4	AAKLLLLKLLLLKLLLLKLLLLK	Complex	mRNA	-3*	0	A549, BEAS-2B, THP-1	2019	[89]
PEG$_{12}$KL4	PEG-AAKLLLLKLLLLKLLLLKLLLLK	Complex	mRNA	-3*	0	A549, BEAS-2B, THP-1	2019	[89]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	mRNA	-8*	10	SKOV-3,	2019	[67]
RALA	WEARLARALARALARHLARALARALRACEA	Complex	mRNA	-0.6*	0	DC2.4	2019	[90]
LAH4	KKALLALALHHLAHLALHLALALKKA	Complex	mRNA	-0.6*	0	DC2.4	2019	[90]
LAH4-L1	KKALLAHALHLLALLALHLAHALKKA	Complex	mRNA	-0.6*	0	DC2.4	2019	[90]
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	Conjugate	mRNA	7	0	DC2.4, RAW246.7, HEK293	2019	[66]
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Conjugate	mRNA	7	0	DC2.4, RAW246.7, HEK293	2019	[66]
C12-H$_5$-S4$_{13}$-PV	Lauroyl-HHHHHALWKTLLKKVLKAPKKKRKVC	Complex	siRNA	50	10–60	U87, HeLa	2020	[51]
H$_5$-S4$_{13}$-PV-C12	Acetyl-HHHHHALWKTLLKKVLKAPKKKRKVCK-Lauroyl	Complex	siRNA	50	10–60	U87, HeLa	2020	[51]
RALA	WEARLARALARALARHLARALARALRACEA	Complex	siRNA	50	0	A549	2021	[91]
HALA1	WEAHLAHALARALARHLARALARALRACEA	Complex	siRNA	50	0	A549	2021	[91]
HALA2	WEARLARALARALARHLARALAHALHACEA	Complex	siRNA	50	0	A549	2021	[91]
HALA3	WEAHLAHALAHALARHLARALARALRACEA	Complex	siRNA	50	0	A549	2021	[91]
HALA4	WEARLARALARALARHLAHALAHALHACEA	Complex	siRNA	50	0	A549	2021	[91]
OligoR	RRRRRRRRR	Complex	mRNA	-4.5*	10	HuH-7	2021	[92]
OligoR-Aib	RRXRRXRRXRRXRRX	Complex	mRNA	-4.5*	10	HuH-7	2021	[92]
Protein transduction domain (PTD)1	PFVYLI	Conjugate	mRNA	-6*	0	K-562	2021	[93]

(*continued on next page*)

Table 1 (*continued*)

CPP	Sequence	Cargo coupling	Cargo	Cargo concentration (nM)	FBS concentration (%)	Cell line	Year	Ref.
PTD2	WSYGLRPG	Conjugate	mRNA	6*	0	K-562	2021	[93]
$(CP)_6$	Cyclo-DPDPDP	Complex	siRNA	100	n.s.	MDA-MB-231	2022	[94]
R_8	RRRRRRRR	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
TAT	GRKKRRQRRRPQ	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
LMWP	VSRRRRRRGGRRRR	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
Stearyl-R_8	Stearyl-RRRRRRRR	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
P5RHH	VLTTGLPALISWIRRRHRRRHC	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
RALA	WEARLARALARALARHLARALARALRACEA	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
Pep-1	KETWWETWWTEWSQPKKKRKV	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
Penetratin	RQIKIWFQNRRMKWKK	Complex	mRNA	6.8	0	CT26.CL25	2022	[65]
Tamra-LK	LKKLLKLLKKLLKLG	Complex	miRNA	50	n.s.	MSC	2022	[68]
Acetyl-LK	Acetyl-LKKLLKLLKKLLKLG	Complex	miRNA	50	n.s.	MSC	2022	[68]
PFC-PR	RRRRR-perfluorocarbon-RGFLGR-perfluorocarbon-RRRRR	Complex	siRNA	100	0, 10	HeLa, HepG2	2023	[95]

The lowest tested cargo concentrations that resulted in a significant effect are specified. The table is sorted by the year of publication. Peptide sequences are specified as single letter amino acid code. Uppercase letters correspond to L-amino acids and lowercase letters correspond to D-amino acids. O: ornithin. X: α-aminoisobutyric acid. FBS: fetal bovine serum. n.s.: not specified. PEG: poly(ethylene glycol). * values calculated based on available data. [1] branched CPP.

case of successful cytoplasmic delivery of the cargo-HiBit proteins.

To compare the potency of the most common CPPs, including TAT, HA2 and R9, the peptides were fused to the GFP-Hibit construct and their cytoplasmic delivery efficiency was quantified. Surprisingly, the highest endosomal escape, which corresponds to the percentage of proteins reaching the cytoplasm after associating with the cell, was found to be achieved by GFP-Hibit without any CPP attached. The measured endosomal escape efficiency of GFP-Hibit was in a range of 1–2 %, while the efficiency for CPPs fused to GFP-Hibit ranged from 0.5 to 1 %. The authors concluded, in line with Lundberg *et al.* in the year 2003 [96], that none of the tested CPPs were actually enhancing endosomal escape of a protein cargo, but rather improved cell surface interaction and, therefore, endocytosis under the applied conditions.

Further indication that CPPs may not efficiently penetrate the endosomal membrane arose from multiple studies using endosomolytic agents to improve the transfection efficiency of their systems. For example Abes *et al.* showed in 2006 that the delivery of DNA-based ASOs, which were fused to cationic peptides, could be markedly increased by simultaneously treating cells with chloroquine [98]. Chloroquine is commonly used to improve the escape of cargos sequestered in the endosomal compartment. The proposed mechanism of action is that chloroquine is protonated in the acidic endosome, leading to the influx of chloride ions and water. This results in swelling of the endosomes and eventual rupture releasing endosomal content into the cytoplasm [99]. Mäe *et al.* observed in 2009 that chloroquine could substantially improve the functional delivery of CPP/RNA complexes by two orders of magnitude [72]. Similar observations were made in other studies with oligonucleotide and mRNA delivery [100–103]. The repeated observation, that chloroquine strongly improved the efficacy of peptide-based nucleic acid transfection, is a strong indicator that endosomal escape remains a crucial bottleneck in the delivery process of payloads, even in the presence of high concentrations of CPPs.

2.3. Cell penetrating peptides applied in vivo

CPPs were also used for nucleic acid delivery *in vivo* [104]. We have selected key peptide-based RNA delivery studies based on the applied CPP and on the availability of adequately detailed transfection protocols and summarized them in Table 2. Prominent CPPs for the delivery of RNA *in vivo* include shGALA [105], NickFect [60,106] and PepFect [67]. As for the *in vitro* transfection studies, the applied amounts of CPP/nucleic acid conjugates or complexes were quite high to obtain a measurable effect. For example in 2019, van den Brand *et al.* used PepFect14 to complex mRNA and transfect ovarian cancer cells [67]. They thoroughly characterized the transfection efficiency of their system in cell culture as well as *in vivo* and provided a comparison to Lipofectamine. However, transfection in cell culture was only assessed under non-challenging conditions with 10 % serum and with an mRNA concentration of 2.68 μg/mL cell culture medium (corresponding to 7.9 nM). This high mRNA concentration resulted in strong transfection. The *in vivo* experiment was performed in mice with an intraperitoneal (i.p.) xenograft tumor. Biodistribution was assessed by quantifying the i.p. administered fluorescently labelled mRNA in different tissues and organs. Interestingly, the authors found that uptake could only be detected in the tumor and not in any of the organs. For analysis of reporter expression, the mice were injected i.p. with 800 μL of complexed mRNA solutions ranging from 2.7 to 8.6 μg of injected mRNA or with a Lipofectamine control. However, for the Lipofectamine control only 2 μg RNA were injected. The authors detected some reporter expression in the outer tumor layers when using PepFect14 nanoparticles with 4.3 μg mRNA or more. The Lipofectamine control did not result in any reporter gene expression. However, it is important to note that also the PepFect14 nanoparticles complexing 2.7 μg mRNA failed to yield any reporter expression in the tumor. Moreover, injecting 800 μL i.p. in mice constitutes a very large volume considering that the mice used in this study weighed around 20 g. Such an injection volume might create

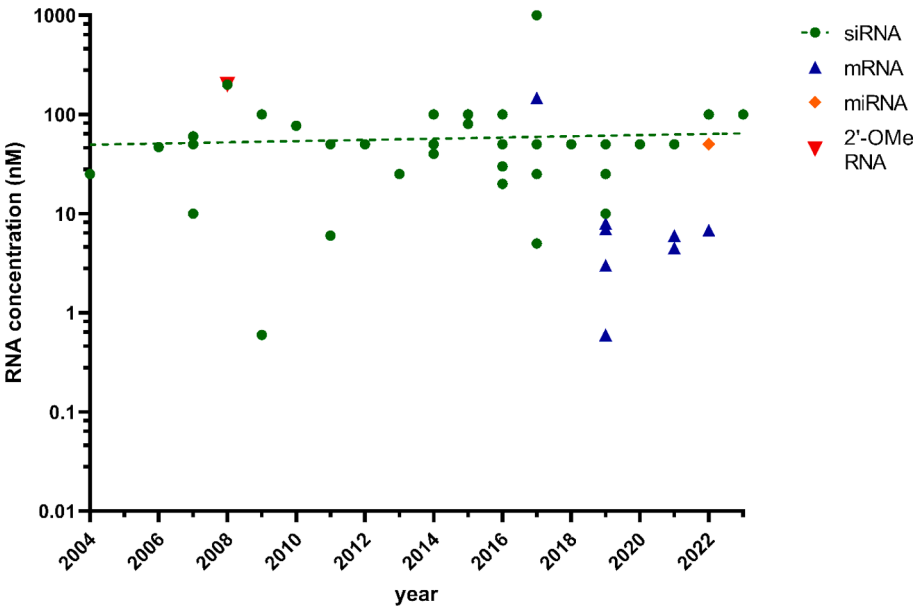

Fig. 1. *In vitro* RNA delivery mediated by CPP-based systems. The lowest tested RNA concentrations that resulted in a significant effect in cell culture experiments are plotted against the respective year of publication. Trendline for siRNA was obtained by nonlinear regression using a semi-log fit. Plotted data is summarized and referenced in Table 1.

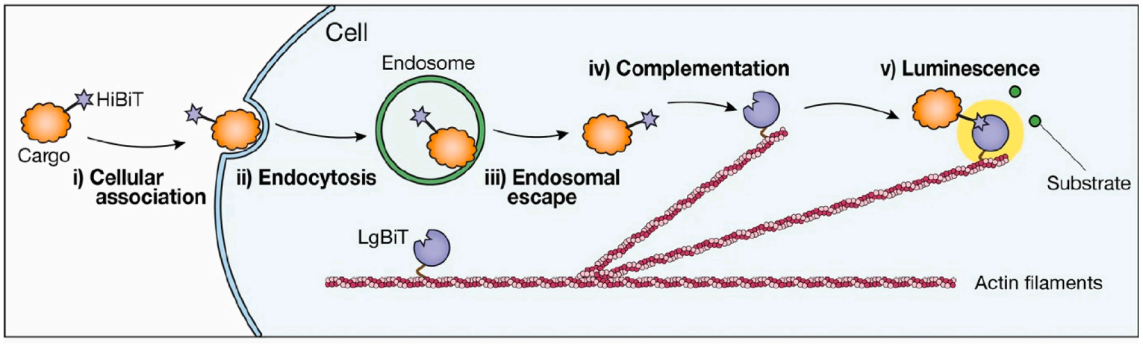

Fig. 2. Schematic illustration of the Split Luciferase Endosomal Escape Quantification (SLEEQ) assay. Endosomal escape is detected by bioluminescence produced after complementation of the split luciferase parts and substrate addition. Figure adapted from Teo et al. [97] under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

significant pressure on the local tissues and the tumor, which could promote cell penetration of the mRNA independently of the peptide [107]. Overall, only a combination of very high mRNA concentrations and injection volumes resulted in some transfection using PepFect14. This study stands exemplary for other CPP-based systems that were used for *in vivo* RNA delivery in the past years. Again, we plotted the lowest tested RNA concentrations from the *in vivo* studies that resulted in a significant effect (Table 2) against the respective year of publication (Fig. 3). The figure indicates that the administered RNA doses did not strongly decrease over the past years, irrespective of the CPP used, the injection route and the delivered RNA species. Hence, also the *in vivo* transfection efficiency is remaining constant at a relatively low level.

2.4. Summary and conclusion on peptide-based endosomal escape

Cationic peptides are frequently used in RNA delivery since they readily complex nucleic acids and promote cell surface association and uptake in low-serum conditions. However, most of the endocytosed RNA is sequestered in the endosomal compartment and does not reach the cytoplasm unless endosomolytic substances, such as chloroquine, are co-administered. In the case of peptide-based protein delivery, less than 1 % of the endocytosed cargo proteins will eventually escape into the cytoplasm by a, to date, unknown mechanism but independently of any of the tested CPPs. Direct measurements of peptide-mediated endosomal escape of nucleic acids are missing, but the fact that such systems require very high doses (*in vitro* and *in vivo*), despite the rather efficient cellular uptake in the absence of serum, indicates that the CPP-mediated endosomal escape of RNA is similarly inefficient as for protein cargo. We conclude therefore that CPPs might not yet be suitable for efficient *in vivo* therapies.

The positive message from the mentioned studies is the observation that endocytosed proteins and nucleic acids can, in fact, escape from the endosome. In the case of protein cargos, the percentage of endosomal escape was consistently quantified at about 1 % of internalized proteins. The mechanism of this escape pathway remains elusive, but could eventually be exploited and amplified to develop improved delivery vectors for nucleic acids.

3. Protein-based endosomal escape enhancers in nucleic acid delivery

Protein-mediated endosomal escape of RNA therapeutics is a rather

Table 2
Summary of key studies using CPP-based systems for the delivery of RNA *in vivo*.

CPP	Sequence	Cargo coupling	Cargo	Cargo dose (μg/mouse)	Injection route	Year	Ref.
Cholesteryl oligo-d-arginine	Chol-RRRRRRRRR	Complex	siRNA	3.5	i.t.	2006	[69]
9R	RRRRRRRRR	Complex	siRNA	50	i.v.	2008	[108]
MPG-8	AFLGWLGAWGTMGWSPKKKRK-cysteamide	Complex	siRNA	1	i.t.	2009	[109]
PepFect6	Stearyl-AGYLLGK(K(K_2(trifluoromethylquinoline$_4$))) INLKALAALAKKIL[1]	Complex	siRNA	25	i.v.	2011	[54]
ShGALA	WEAALAEALAEALAEHLAEALA	Complex	siRNA	100	i.v.	2011	[105]
PLL-CA	Poly(L-lysine-cholic acid)	Complex	siRNA	25	i.v.	2012	[76]
cRGD-PEG-*b*-PLL	Cyclo-RGD-(PEG-*block*-poly(L-lysine))	Complex	siRNA	24	i.v.	2012	[110]
$CH_2R_4H_2C$	CHHRRRRHHC	Complex	siRNA	25	i.v.	2013	[111]
PLG*LAG-R_9	PLGLAGRRRRRRRRR	Complex	siRNA	20	i.v.	2014	[112]
C6M1	Acetyl-RLWRLLWRLWRRLWRLLR	Complex	siRNA	4	i.t.	2014	[113]
STR-HK	Stearyl-HHHPKPKRKV	Complex	siRNA	4	i.t.	2015	[114]
599	GLFEAIEGFIENGWEGMIDGWYGGGGRRRRRRRRRK	Complex	siRNA	5	i.t.	2015	[115]
PPABLG	poly(γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl-l-glutamate)	Complex	siRNA	1.25	i.v.	2016	[116]
TAT	RKKRRQRRRC	Complex	siRNA	3.5	i.v.	2016	[117]
OA-R_8	Oleoyl-RRRRRRRR	Complex	siRNA	62.5	i.v.	2016	[118]
CL	KVRVRVRVpPTRVRERVK	Complex	siRNA	50	i.v.	2017	[119]
R_{16}-hepCPP	RRRRRRRRRRRRRRRRKRPTMRFRYTWNPMK	Complex	siRNA	16.75	i.v.	2017	[120]
BFPD	Crosslinked fluorinated poly(L-lysine) dendrimers	Complex	siRNA	34	i.t.	2017	[121]
DRI	rrrrrrrrr	Complex	siRNA	20	i.v.	2017	[122]
NickFect70	Arachidyl-HHHHYHHGOδILLKALKALAKAIL	Complex	siRNA	40	i.v.	2019	[60]
PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL	Complex	mRNA	4.3	i.p.	2019	[67]
AmPPDs	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-poly(L-lysine) dendrimers	Complex	siRNA	75	i.t.	2020	[123]
PHD / PLL	PEG-poly(L-histidine)-poly(sulfadimethoxine) / poly(L-lysine)	Complex	siRNA	25	i.v.	2020	[124]
R_8-bola	RRRRRRRR-dodecyldiamine	Complex	siRNA	25	i.v.	2020	[125]
p5RHH	VLTTGLPALISWIRRRHRRHC	Complex	siRNA	12.5	i.v.	2020	[126]
MPEG-PCL-CH2R4H2C	(methoxyPEG-*block*-poly(ε-caprolactone))-CHHRRRRHHC	Complex	siRNA	20	i.v.	2020	[127]
T7-PEG-SHRss	HAIYPRH-PEG-HH(H-stearyl)CRRRRRC[1]	Complex	siRNA	50	i.v.	2021	[128]
APNPs	(H-Cys-SH)$_2$-lys-Glu(G2)-Obzl[1]	Conjugate	siRNA	5	i.v.	2021	[129]
MPEG-PCL-CH2R4H2C	(methoxyPEG-*block*-poly(ε-caprolactone))-CHHRRRRHHC	Complex	siRNA	20	i.v.	2022	[130]
PEG-CPP33	PEG-RLWMRWYSPRTRAYG	Complex	siRNA	37.5	i.v.	2023	[131]
NickFect424	Stearyl-AGYLLGD$_{ab}$LKALAALAKAIL	Complex	mRNA	62.5	i.v.	2023	[106]
NickFect436	Stearyl-AGYLLGD$_{ab}$LKALAALAAKIL	Complex	mRNA	62.5	i.v.	2023	[106]

The lowest tested cargo doses that resulted in a significant effect are specified. The table is sorted by the year of publication. Peptide sequences are specified as single letter amino acid code. Uppercase letters correspond to L-amino acids and lowercase letters correspond to D-amino acids. O: ornithin. D_{ab}: 2,4-diamonobutyric acid. PEG: poly(ethylene glycol). i.v.: intravenous. i.t.: intratumoral. i.p.: intraperitoneal. [1] branched CPP.

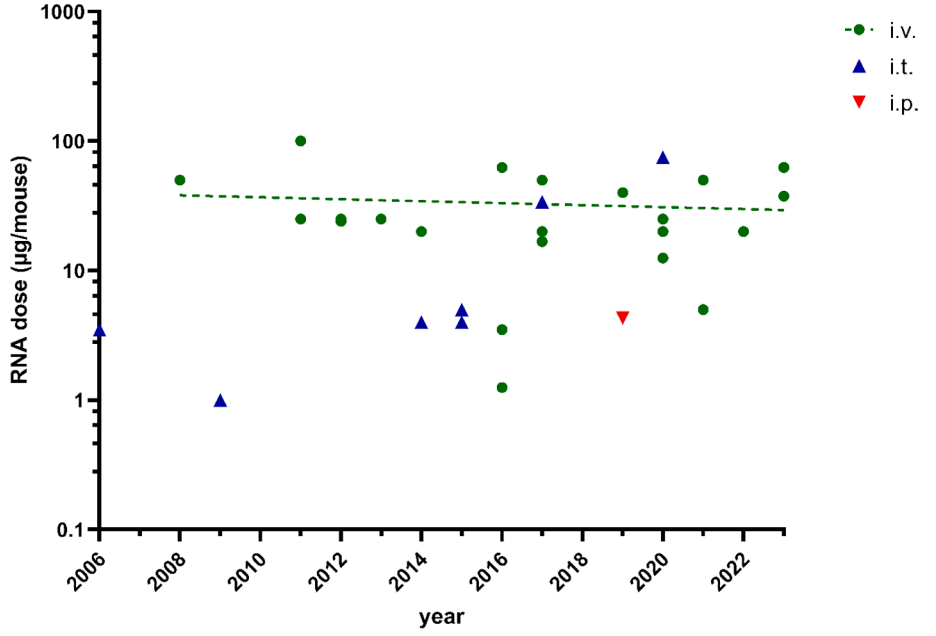

Fig. 3. *In vivo* RNA delivery mediated by CPP-based systems. The lowest tested RNA doses which were effective *in vivo* are plotted against the respective year of publication. i.v.: intravenous injection. i.t.: intratumoral injection. i.p.: intraperitoneal injection. Trendline for i.v. was obtained by nonlinear regression using a semi-log fit. Plotted data is summarized and referenced in Table 2.

unexplored field of research, especially compared to research conducted on CPPs or cationic lipids. It is essential to understand the differences between protein- and peptide-based endosomal escape enhancers in order to assess which system might be superior for enhancing endosomal escape. Proteins have the great advantage of being able to exert distinct functions very efficiently due to specific secondary and tertiary structures [132]. There are, for example, many specialized pathogenic proteins that enable efficient pore formation in the endosomal compartment. Other proteins display highly evolved lipase activity, which allows them to degrade endosomal membranes. There are, to the best of our knowledge, no pathogens which escape the endosomes solely with the help of peptides. Nevertheless, the drawback of using proteins as endosomal escape enhancers for drug delivery is their often limited structural stability in body fluids and immunogenicity [31,34,133,134]. Moreover, it might be more difficult to deliver endosomal escape-promoting proteins together with a therapeutic payload, since proteins might denature during the production or storage of the formulation. Considering that comparably little research was conducted on proteins as endosomal escape enhancers for non-viral RNA therapeutics, we will discuss protein-based endosomal escape with a stronger focus on the delivery of nucleic acids in general and not only on RNA therapeutics. The protein-based endosomal escape enhancers discussed in this chapter are summarized in Table 3. Moreover, the lowest tested nucleic acid concentrations that resulted in a significant cellular effect (Table 3) are plotted against the respective year of publication (Fig. 4). The average nucleic acid concentration required to achieve an effect *in vitro* lies in the low nM range (roughly around 1 nM). This value is, therefore, around 100 fold lower compared to CPP-mediated nucleic acid delivery *in vitro* (Fig. 1).

3.1. Pore-forming proteins in nucleic acid delivery

An RNA delivery system containing a pore-forming protein for escaping the endosomal compartment was developed by Liu *et al.* in 2014 [135]. This system was established for siRNA delivery and made use of recombinant fusion proteins. The first fusion protein comprised an RNA-binding domain coupled to a domain targeting epidermal growth factor receptor (EGFR) on the cell surface. Transfection of GFP-specific siRNA using homodimers of the RNA-binding and EGFR-targeting fusion protein resulted in uptake of fluorescently labelled siRNA but failed to produce significant knockdown of GFP *in vitro*. Microscopy analysis revealed the entrapment of labelled siRNA in the endosomal compartment, due to the absence of an endosomal escape enhancer. Therefore, the authors of the study included a second protein to promote endosomal escape; the pore-forming protein perfringolysin O (PFO). PFO is a cholesterol-dependent cytolysin and is secreted by the pathogen *Clostridium perfringens* (*C. p.*). It ultimately forms pores in cholesterol-rich membranes by membrane binding, oligomerization, pre-pore formation and eventually formation of the mature pore with an inner diameter ranging from 25 to 30 nm. Therefore, the pore might be wide enough to allow diffusion of small RNA species, such as siRNAs. Membrane binding as well as pore formation is enhanced at acidic pH [146,147]. Liu *et al.* fused PFO to the EGFR-targeting domain of their delivery protein. Eventually, GFP knockdown could be achieved when using PFO together with the RNA-binding protein, which were both fused to the EGFR-targeting domain. A non-targeted PFO fusion protein failed to reduce GFP expression meaning that EGFR targeting and internalization of PFO was required for siRNA transfection. Therefore, the authors concluded that PFO might enable cytoplasmic delivery of siRNA *via* escape from endosomes and not *via* pore formation in the plasma membrane. However, significant cytotoxicity was observed at PFO fusion protein concentrations above 100 pM. To further improve their system, the authors introduced a third fusion protein harboring EGFR binding domains that cluster EGFR on the plasma membrane, which is according to the study thought to increase internalization. The final delivery system consisted of three combined fusion proteins exhibiting the functionalities of RNA binding, EGFR targeting, EGFR clustering as well as inducing uptake and endosomal escape. With this system, more than 50 % GFP knockdown could be achieved under serum-free conditions with 100 pM of the endosomal escape promoting PFO and 16 nM siRNA. The limitation of this study is, however, the toxicity of PFO at elevated concentrations resulting in a narrow therapeutic window, which might be especially problematic *in vivo*. Another major drawback is that PFO was not directly coupled with the RNA in a stable complex but targeted separately from the siRNA to the cell surface. While this was sufficient to generate transfection in a cell culture setting with long transfection times, the system would probably not work *in vivo*, as long as the RNA and the PFO are not stably coupled. Whether PFO would also promote endosomal escape if complexed with the RNA, *e.g.* in an RNA nanoparticle, remains to be assessed. Also, the delivery of larger RNA species, such as mRNA, was not demonstrated, and it is currently unclear whether PFO is establishing a stable pore in the endosomal membrane, or rather results in endosome rupture. Moreover, it is currently unclear if PFO could also enhance delivery of siRNA by directly forming pores in the plasma membrane.

The above-mentioned system was further improved in follow-up studies by the same group [148]. In the publication by Yang *et al.*, yeast surface display yielded a protein binder based on a fibronectin scaffold, which is able to reversibly neutralize the membranolytic activity of PFO. This binder was engineered to bind and inhibit PFO at

Table 3
Protein-based endosomal escape enhancers used for gene delivery.

Endosomal escape enhancer	Origin	Proposed mechanism	Cargo coupling	Cargo	Cargo concentration (nM)	FBS concentration (%)	Cell line	Year	Ref.
Perfringolysin O	*C. f.*	Pore formation	None	siRNA	16	0	A431	2014	[135]
Perfringolysin O	*C. f.*	Pore formation	None	siRNA	0.23	10	A431	2017	[136]
Listeriolysin O	*L. m.*	Pore formation	Complex	DNA	~0.28*	0	Huh7, SK Hep 1	1999	[137]
Listeriolysin O	*L. m.*	Pore formation	Complex	DNA	~0.62*	10	HEK293, RAW264.7, P388D1	2003	[138]
Listeriolysin O	*L. m.*	Pore formation	Encapsulation	ASOs	500	10	Bone-marrow macrophages	2003	[139]
Listeriolysin O	*L. m.*	Pore formation	Encapsulation	DNA	~2.2*	5	P388D1	2005	[140]
Listeriolysin O	*L. m.*	Pore formation	Complex	DNA	~0.4*	0	HEK293	2008	[141]
Listeriolysin O	*L. m.*	Pore formation	Complex	DNA	~1.4*	0, 10	P388D1	2014	[142]
Phospholipase A_2	*A. m.*	Phospholipid hydrolysis	Complex	DNA	~1.1*	0	HEK293, HepG2	2010	[143]
Phospholipase A_2	*A. m.*	Phospholipid hydrolysis	Dispersion	DNA	~0.3*	0	COS7	2011	[144]
Phospholipase C	*L. m.*	Phospholipid hydrolysis	Complex	DNA	0.06	10, 99	HeLa	2022	[145]

The lowest tested cargo concentrations that resulted in a significant effect are specified. The table is sorted by the type of endosomal escape enhancer. C. f.: Clostridium perfringens. L. m.: *Listeria monocytogenes*. A. m: Apis mellifera (honeybee). FBS: fetal bovine serum. * values calculated based on available data.

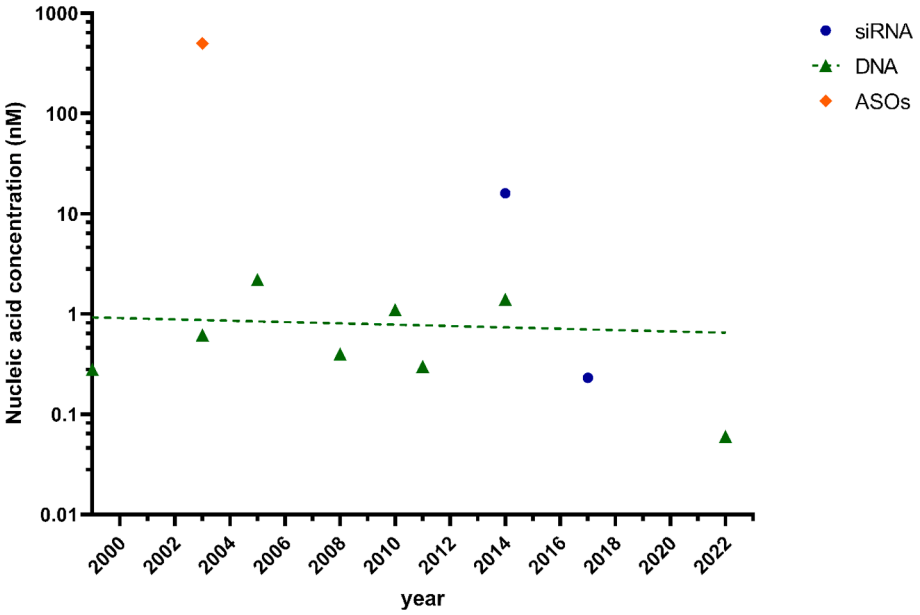

Fig. 4. *In vitro* nucleic acid delivery mediated by protein-based systems. The lowest tested nucleic acid concentrations that resulted in a significant effect in cell culture experiments are plotted against the respective year of publication. Trendline for DNA was obtained by nonlinear regression using a semi-log fit. Plotted data is summarized and referenced in Table 3.

neutral pH, but to release PFO in the acidic environment of the endosome (Fig. 5). This resulted in a more stringent activation of PFO in the endosomal compartment.

This system is thought to mainly allow pore formation in the endosomal compartment and to reduce it in the plasma membrane, since the major fraction of PFO in the extracellular space should be neutralized by the binder. Therefore, with this adapted system the cytotoxicity, which was assessed with a hemolysis assay, could be drastically reduced while the transfection efficiency was retained. This optimized PFO-based endosomal escape enhancer was then used to improve GFP silencing by delivery of siRNA with an adapted system containing a different RNA-binding protein [136]. In this more advanced system 230 pM siRNA together with 5 nM adapted PFO-based endosomal escape enhancer were needed for achieving 50 % GFP knockdown. Hence, including the neutralizing PFO binder together with EGFR targeting moieties resulted in potent gene silencing together with non-detectable cytotoxicity. However, other major limitations remained in this system. PFO is still not attached to the RNA and the system was tested by transfecting cells for 6 h in cell culture media supplemented with 10 % serum. Testing the system under more challenging conditions, such as shorter incubation time and higher serum content, would give valuable insights on the potential of the system for *in vivo* applications. Summarizing, when intending to use PFO as endosomal escape enhancer in combination with other systems, one should consider that targeting of PFO to receptors on the plasma membrane might be essential, as observed in this study and the study by Liu *et al.* [135]. In these studies EGFR targeting was necessary for cytoplasmic delivery. Nevertheless, we could imagine that targeting another receptor than EGFR would also result in efficient delivery of cargo to the cytoplasm. Moreover, inhibition of PFO pore formation at neutral pH enabled by binding proteins should be considered in order to reduce cytotoxicity and to obtain a wider therapeutic window. It must additionally be stated that PFO was shown to be immunogenic in mice. PFO is indeed investigated as candidate for the development of vaccines against *C. p.* [149,150].

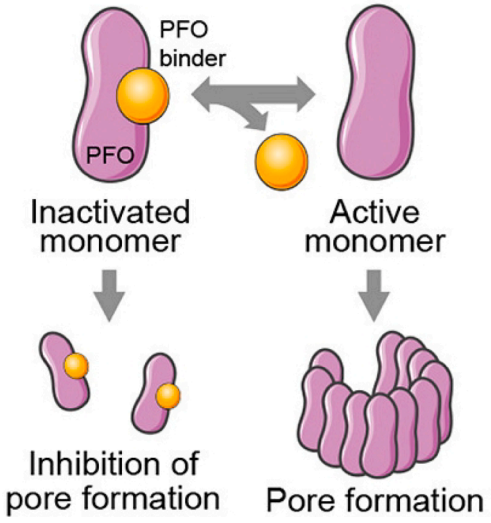

Fig. 5. Schematic illustration of PFO pore formation dependent on dissociation of inhibiting PFO binder. The PFO binder is designed to bind PFO at neutral pH, as encountered in the extracellular space, and therefore inhibit pore formation. At acidic pH, as encountered in endosomes, the PFO binder is designed to dissociate allowing PFO pore formation. Reprinted with permission from Yang *et al.* [148] Copyright 2015 American Chemical Society.

A similar pore-forming protein that was used for endosomal escape of nucleic acids is listeriolysin O (LLO), which is secreted by *Listeria monocytogenes (L. m.)* together with phospholipases to escape the phagosomes during infection. LLO belongs, as PFO, to the cholesterol-dependent cytolysins and is most active at acidic pH. The pores formed by LLO have the same diameter as the pores formed by PFO, *i.e.* ca. 25–30 nm [151,152]. In 1999, Walton *et al.* used LLO together with a cell type specific targeting and DNA complexation moiety for delivering luciferase-encoding plasmid DNA (pDNA) into cells [137]. Their system contained asialoglycoprotein-poly(L-lysine) conjugates for complexing DNA as well as targeting hepatic asialoglycoprotein receptors. The authors transfected cells in DMEM without serum for 4 h using 1 μg of pDNA per mL of medium. Hence, the chosen conditions were rather unchallenging and used large amounts of DNA. Nevertheless, transfection experiments revealed that the chemical conjugation of LLO to the poly(L-lysine) was essential for obtaining transfection, as assessed by bioluminescence produced by luciferase expression. Interestingly, transfection efficiency decreased by around 60 % when cell membrane

targeting of the complexes was competed with free targeting moieties. Moreover, adding free LLO, *i.e.* without targeting and DNA binding moiety, to the cells together with the DNA complexes was not sufficient for effective transfection. The fact that targeting of LLO to cells is required for efficient transfection was also observed for PFO-based endosomal escape constructs, as described in the section above.

The ability of LLO to enable efficient nucleic acid transfection was further confirmed by other studies using different nucleic acid carriers, such as protamine for DNA complexation [138,140,142], liposomes for ASOs encapsulation [139] or PEI for DNA complexation [141]. The studies using pDNA have in common, that quite large amounts of cargo are used for transfecting cells. However, DNA plasmids are large molecules with several thousand bp. This size difference must be considered when comparing concentrations of DNA and RNA. Moreover, DNA must reach the nucleus for efficient transfection, which constitutes another hurdle not encountered by most RNA-based therapeutics. Further, the LLO studies mentioned here are forming the DNA/LLO complexes with an excess of LLO protein, which results in some free LLO during the cell transfection experiments. The influence of this free LLO species (fused to PEI, poly(L-lysine) or membrane targeting factors) has not been evaluated but could be substantial. Hence, it remains to be demonstrated, to what degree complexed LLO is able to promote endosomal lysis or whether it would require a mechanism of endosomal LLO release to trigger endosomal escape.

To conclude, LLO might present a promising endosomal escape agent for the delivery of nucleic acids. However, the efficiency should still be enhanced, considering that rather high amounts of cargo were used in the studies described above. The potential cytotoxicity of higher LLO concentrations must be considered. To prevent this, systems with decreased pore formation at neutral pH, similar to the mechanisms described for PFO above, could be developed. Moreover, it is important to note that LLO was shown to be strongly immunogenic, [153] which would likely limit its *in vivo* applications as endosomal escape enhancer if chronic administration is required.

3.2. Phospholipases in nucleic acid delivery

Besides using pore-forming proteins, another protein-based strategy to enhance endosomal escape is the use of phospholipases. However, literature on using phospholipases for enhancing delivery of nucleic acids is very scarce indicating that not much research was conducted on this approach. One example is Le *et al.*, who used bee venom phospholipase A_2 (PLA_2) conjugated to PEI for enhancing delivery of pDNA encoding for luciferase or GFP [143]. PLA_2 is an acyl esterase and hydrolyzes glycerophospholipids at the *sn*-2 position (Fig. 6) [154]. Transfection was performed by incubating the cells for 3 h with the complexes under serum-free conditions, using 1 μg DNA per well in a 24 well plate. Transfection efficiency was assessed by luciferase activity or GFP expression. The authors could show that including PLA_2 resulted in increased transfection when higher N/P ratios were used. Again, under these conditions a considerable fraction of the PLA_2-PEI would be present in an uncomplexed form and could largely influence the transfection of the particles. However, this issue was not addressed by the authors.

The ability of bee venom PLA_2 to enhance transfection was also shown by Toita *et al.* [144]. This study reported a delivery system consisting of PLA_2 incorporated together with pDNA into a polysaccharide nanogel. Assessing transfection efficiency by bioluminescence analysis revealed that using PLA_2 concentrations of 50 nM or higher resulted in increased transfection. Interestingly, addition of PLA_2 did not increase uptake into cells, as assessed by flow cytometry analysis of fluorescently labelled complexes. The authors hypothesized that PLA_2 enhances transfection by hydrolyzing endosomal membrane lipids resulting, eventually, in escape from endosomes and release of complexes into the cytoplasm.

Another approach which uses a phospholipase for enabling efficient transfection was developed recently by our group [145]. In this approach, pDNA was complexed with human mitochondrial transcription factor A (TFAM) and combined with phosphatidylcholine-specific phospholipase C (PLC). The PLC was derived from *L. m.* and is a virulence factor which contributes to pathogen escape from the phagosome into the cytoplasm. Moreover, PLC shows maximal activity at acidic pH and is active on a broad range of phospholipids and cleaves phospholipids by hydrolyzing their phosphodiester bond (Fig. 6) [155]. Complexes formed by TFAM and PLC were able to efficiently transfect cells as assessed by flow cytometry analysis of GFP expression. Importantly, transfection was abolished when an inactive PLC variant or no PLC was used. Moreover, the final system achieved high transfection under challenging conditions, *i.e.* low DNA concentrations of 200 ng/mL (60 pM) and transfection in 99 % serum on a confluent cell layer. This indicates that PLC might be a powerful endosomal escape enhancer. However, also in this system the contribution of complexed *versus* free enzyme on the transfection efficiency is yet to be characterized.

In conclusion, PLC enables this system to efficiently transfect cells *in vitro*, even under challenging conditions. Our group is currently working on improving this delivery system before it will be tested *in vivo* in the near future. One point to consider is that this system is restricted to delivery of DNA, since TFAM is not known to condense RNA into nanoparticles. Nevertheless, PLC can readily be translated as promising endosomal escape enhancer to other protein-based nucleic acid delivery systems, for example covalently by genetic fusion or non-covalently by co-administration. It could, for example, be combined with RNA binding domains and therewith enable RNA delivery.

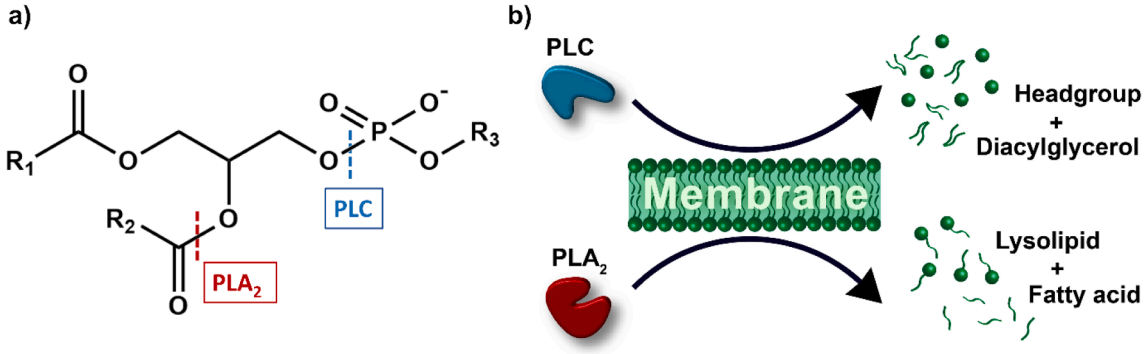

Fig. 6. Phospholipid hydrolysis by phospholipase A_2 (PLA_2) and phospholipase C (PLC). (a) PLA_2 hydrolyzes the ester bond of the fatty acid at the sn2 position. PLC hydrolyzes the phosphodiester bond connecting the phosphate group to the glycerol backbone. (b) Schematic illustration of membrane hydrolysis mediated by PLA_2 and PLC. PLA_2 cleaves phospholipids resulting in lysolipids and fatty acids while phospholipid cleavage mediated by PLC results in free phospholipid headgroups and diacylglycerol molecules.

3.3. Summary and conclusion on protein-based endosomal escape

Pore-forming proteins and phospholipases from venom or bacterial pathogens are highly effective in penetrating the endosomal or plasma membranes and therefore can enable efficient delivery of cargo. The average *in vitro* applied nucleic acid dose is around 100 fold lower for protein-based delivery systems (Table 3 and Fig. 4) when compared to CPP-based systems used for nucleic acid delivery (Table 1 and Fig. 1). However, delivery systems including the above-mentioned proteins might be immunogenic, which constitutes the obvious downside exhibited by those endosomal escape factors. Evidence for immunogenicity was shown for pore-forming proteins [149,153] as well as venom [156] and bacterial phospholipases [157,158]. Nevertheless, potential immunogenicity of such pathogenic endosomal escape proteins should be assessed for the entire delivery system and not for the protein moiety alone. To reduce immunogenicity, human homologues of these pathogenic proteins could be tested for endosomal escape, such as the human pore-forming protein perforin [159] or human phospholipases [160].

4. General conclusion and perspectives

In this review, we discussed the current state of peptide- and protein-mediated endosomal escape regarding the delivery of nucleic acids. We summarized the concepts and physical properties of the various nucleic acid-based therapeutics, the destination in the cell where they exert their functions and why their delivery is so problematic.

In the past decades, various CPP variants were developed with the aim to increase endosomal escape of nucleic acids. Unfortunately, only a few research groups have attempted to directly quantify the endosomal escape enhancing effect of these entities. Those who did, could not detect a reproducible effect on endosomal escape and attributed the transfection efficiencies of some peptides to enhanced cell surface binding and endocytosis. This aligns with the observation that all current CPP-based RNA delivery systems require high concentrations of RNA in order to be effective *in vitro*. Further, the CPP-based systems are usually developed and tested in cell culture conditions without serum or with only 10 % serum. Higher serum concentrations, for example 100 % serum as encountered in the blood, would interfere with the charge specific uptake of the CPPs and further diminish their potency. This also explains why the RNA doses used in CPP-based *in vivo* experiments are very high.

Protein-based endosomal escape is not as popular as escape mediated by peptides, which might be owed to the fact that proteins are more challenging to produce and their incorporation into nanoparticles is difficult. However, clear evidence exists that specialized proteins are in fact able to penetrate biological membranes with high efficiency. Unfortunately, the proteins which have been used for this purpose to date are originating from pathogens or venoms, and might, therefore, exhibit immunogenicity which must be considered when using these factors for RNA delivery *in vivo* [134,149,153,156]. The future perspective of protein-based endosomal escape enhancers could be the use of human homologues of the pathogenic endosomal escape-enhancing enzymes, such as human pore-forming proteins [159] and phospholipases [160]. These homologues could be used to develop highly potent as well as safe endosomal escape agents.

We would like to conclude this article by suggesting a set of standard conditions for the assessment and comparability of the efficiency of novel transfection agents. Adhering to the three points outlined below would, in our opinion, support the stepwise development of more potent nucleic acid delivery vectors for *in vivo* applications. First, novel systems, especially the ones intended to be injected in the vascular system, should be tested on a nearly confluent cell layer in more than 90 % serum to partially mimic an *in vivo*-like situation. Second, the systems should be incubated on the cells for up to one hour but not longer. Third, the EC50 values with regard to the nucleic acid concentration should be always determined and specified in order to allow the comparison of different systems as well as to reliably assess the improvement in transfection efficiency. To further simplify comparisons, we additionally strongly recommend to always indicate the transfection conditions as detailed as possible (*e.g.* type of cell culture plate used and applied volume of cell culture media). We believe that relevant and comparable assay conditions are crucial in the development of better transfection systems, which might eventually contribute to overcoming one of the major hurdles of nucleic acid delivery, the endosomal escape.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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