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Efficient Intracellular Delivery of Nucleic Acid Pharmaceuticals Using Cell-Penetrating Peptides

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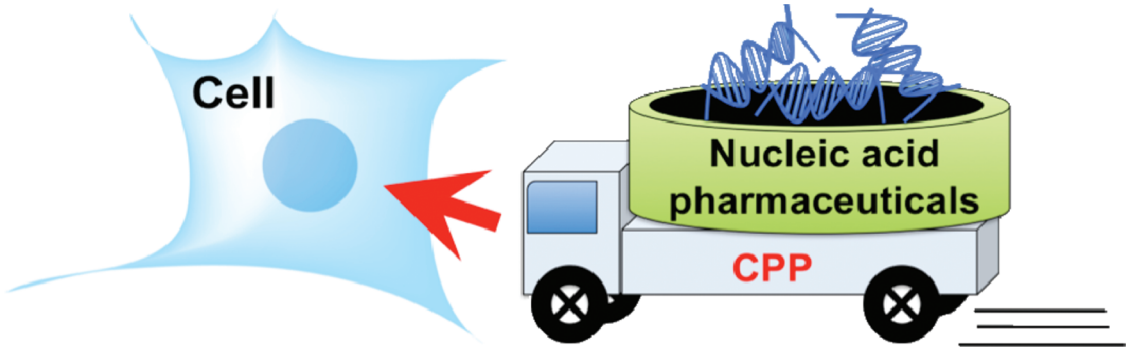

Over the last 20 years, researchers have designed or discovered peptides that can permeate membranes and deliver exogenous molecules inside a cell. These peptides, known as cell-penetrating peptides (CPPs), typically consist of 6−30 residues, including HIV TAT peptide, penetratin, oligoarginine, transportan, and TP10. Through chemical conjugation or noncovalent complex formation, these structures successfully deliver bioactive and membrane-impermeable molecules into cells. CPPs have also gained attention as an attractive vehicle for the delivery of nucleic acid pharmaceuticals (NAPs), including genes/plasmids, short oligonucleotides, and small interference RNAs and their analogues, due to their high internalization efficacy, low cytotoxicity, and flexible structural design.

In this Account, we survey the potential of CPPs for the design and optimization of NAP delivery systems. First, we describe the impact of the N-terminal stearylation of CPPs. Endocytic pathways make a major contribution to the cellular uptake of NAPs. Stearylation at the N-terminus of CPPs with stearyl-octaarginine (R8), stearyl-$(RxR)_4$, and stearyl-TP10 prompts the formation of a self-assembled core−shell nanoparticle with NAPs, a compact structure that promotes cellular uptake. Researchers have designed modifications such as the addition of trifluoromethylquinoline moieties to lysine residues to destabilize endosomes, as exemplified by PepFect 6, and these changes further improve biological responsiveness. Alternatively, stearylation also allows implantation of CPPs onto the surface of liposomes. This feature facilitates "programmed packaging" to establish multifunctional envelope-type nanodevices (MEND). The R8-MEND showed high transfection efficiency comparable to that of adenovirus in non-dividing cells.

Understanding the cellular uptake mechanisms of CPPs will further improve CPP-mediated NAP delivery. The cellular uptake of CPPs and their NAP complex involves various types of endocytosis. Macropinocytosis, a mechanism which is also activated in response to stimuli such as growth factors or viruses, is a primary pathway for arginine-rich CPPs because high cationic charge density promotes this endocytic pathway. The use of larger endosomes (known as macropinosomes) rather than clathrin- or caveolae-mediated endocytosis has been reported in macropinocytosis which would also facilitate the endocytosis of NAP nanoparticles into cells.

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1. Introduction

Around 20 years ago, the ability of the human immunodeficiency virus (HIV)-1 Tat and Antennapedia homeodomain proteins to penetrate cells was discovered.[1] Subsequent studies have shown that short, basic peptides corresponding to the RNA-/DNA-binding domains of these proteins were responsible for their translocation into cells, and that the conjugation of various exogenous proteins with these basic peptides allowed their efficient delivery into cells.[2,3] Considerable research has focused on the development of peptides known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs), which enable the intracellular delivery of bioactive molecules with low membrane permeability.[3−5] In addition to the above-mentioned peptides derived from HIV-1 Tat (TAT peptide)[6] and Antennapedia homeodomain protein (penetratin),[7] oligoarginine,[2,8] transportan,[9] and TP10[10] are the most widely employed CPPs (Figure 1). Using these CPPs, various bioactive and membrane-impermeable molecules possessing different physicochemical properties and molecular weights/sizes have successfully been delivered into cells. One interesting recent application concerns the use of a CPP to enable the introduction of stable isotope-labeled proteins into eukaryotic cells for the investigation of their in vivo conformation and dynamic properties by so-called in-cell NMR.[11]

On the other hand, recent developments in molecular biology and genome science have led to the discovery of a number of disease-related genes. Attempts to apply these findings to the therapy of genetic and acquired diseases, including cancer and viral diseases, are in progress. In addition to gene delivery and antisense therapies, RNA interference (RNAi) is a promising therapeutic methodology. Nucleic acid pharmaceuticals (NAPs) target intracellular molecules; therefore, the development of efficient methods for the intracellular delivery of NAPs, including genes/plasmids, short oligonucleotides (ONs), and small interfering RNAs (siRNAs), and their analogues is necessary.

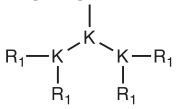
CPPs have received attention as an attractive means for NAP delivery, not only because of their high internalization ability but also due to their potential for variable structural design. Significant improvements in the design and performance of CPP-based NAP delivery systems have been made in the past few years (see refs 12−15 and those cited therein). In this Account, we summarize current CPP-based NAP delivery approaches, especially self-assembling core−shell NAP nanoparticles comprising stearylated CPPs, and those employing liposome-based multifunctional envelope-type nanodevices (MEND). Their impact on NAP delivery and therapeutic potential is also discussed.

CPPs

HIV-1 TAT:	GRKKRRQRRRPPQ
R8:	RRRRRRRR
penetratin:	RQIKIWFQNRRMKWKK
Transportan:	GWTLNSAGYLLGKINLKALAALAKKIL-amide
TP10:	AGYLLGKINLKALAALAKKIL-amide

STR-CPPs

STR-R8:	stearly-RRRRRRRR-amide
STR-$(RxR)_4$:	stearly-$(RxR)_4$-amide (x=6-aminohexanoic acid)
STR-TP10:	stearyl-AGYLLGKINLKALAALAKKIL-amide
PF6:	stearyl-AGYLLGKINLKALAALAKKIL-amide



PF14:	stearyl-AGYLLGKLLOOLAAAALOOLL-amide (O=ornithine)
NickFect1:	stearyl-AGY(PO_3)LLGKTNLKALAALAKKIL-amide

stearyl = $CH_3(CH_2)_{16}CO$-

R_1 =

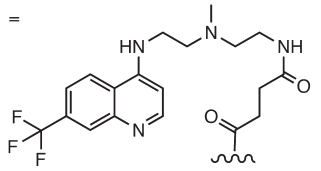


FIGURE 1. Structures of representative CPPs and stearylated CPPs (STR-CCPs).

2. NAP Delivery Using Stearylated CPPs

The original reports on TAT and other CPPs described their potential for the delivery of peptides and proteins into cells.[2−4,6−10,16] The CPPs and their peptide/protein conjugates were internalized into nearly 100% of cells in a few to several tens of minutes and yielded the bioactivity of the intracellularly delivered peptides/proteins; however, covalent protein−CPP conjugation was required for effective delivery. Inspired by these results, attempts have been made to utilize CPPs for the delivery of NAPs (e.g., gene, ON, and siRNA) into cells. These approaches can be divided into two types: the conjugation of NAPs with arginine-rich CPPs and the formation of noncovalent complexes of CPPs and NAPs. However, considering the need for specific conjugation procedures and the easy formation of precipitates of cationic CPPs and negatively charged NAPs, the latter approach is preferred for gene and nucleic acid delivery.

Although reports have described the transfection of plasmid DNAs (pDNAs) into cells in complexes with TAT itself or other unmodified CPPs, delivery was inefficient.[14,17]

Although these approaches yielded somewhat greater gene expression than control (plasmid only) transfection, the improvement in transfection efficiency was not as great as with cationic liposomes (e.g., Lipofectamine) or polymers (e.g., polyethyleneimine).

To improve transfection efficiency, Futaki et al.[18] evaluated the effect of the introduction of a hydrophobic moiety to arginine-rich CPPs. The interaction of these hydrophobic moieties with each other or with nucleobases in the plasmid/CPP complexes may enhance compaction of the pDNA and lead to self-assembling particle formation that will favor NAP delivery in vitro and in vivo. The increased hydrophobicity of the complex may enhance cell surface adsorption and so aid in membrane translocation.

A comparison of the transfection efficiency of N-terminally acylated oligoarginines with variable numbers of arginines and acyl chains, stearyl-octaarginine (STR-R8) was found to have the highest transfection efficiency.[18] Simple mixing of STR-R8 with pDNA yielded a transfection efficacy comparable to that of Lipofectamine with no significant cytotoxicity.[18,19] Atomic force microscopy revealed the effective condensation of pDNA by STR-R8, but not R8, to yield particle-like structures having diameters of ~120 nm and a positive zeta potential (19.3 mV).[19] Therefore, the stearyl moiety in arginine-rich CPPs acts as a hydrophobic core to facilitate the formation of particle structures while maintaining a positive surface charge. This may also lead to enhanced adsorption on cell surfaces and eventual cellular uptake.

STR-R8 was also employed for siRNA delivery. Tönges et al.[20] reported enhanced siRNA transfection into primary hippocampal neurons and the effective induction of siRNA-mediated gene silencing in primary neuron cultures with lower cytotoxicity than a cationic liposome-based transfection agent. A similar approach was reported by Kim et al.,[21] who employed nonaarginine (R9) modified with cholesterol. The siRNA-containing noncovalent cholesteryl-R9 complex targeting vascular endothelial growth factor (VEGF) was not just effective in vitro; local administration in vivo led to tumor regression.

N-Terminal acylation for the compaction of DNA/RNA complexes to stimulate cellular uptake may be applicable to other types of CPPs. TP10 is another representative CPP with a primary amphiphilic, hydrophobic/weakly basic structure developed by Langel and his colleagues.[10] Although the physicochemical properties of this CPP are somewhat different from those of arginine-rich CPPs, the N-terminal stearylation of TP10 (STR-TP10) markedly improved the delivery efficiency of a phosphorothioate 2′-*O*-methyl RNA oligonucleotide (2′-OMe ON) for splice correction into cells.[22] Aberrant pre-mRNA splicing is involved in various diseases, and the blockage of aberrant splice sites by ON derivatives has significant therapeutic potential. However, effective methods for the intracellular delivery of ON derivatives are required to obtain therapeutic effects. The splice correction levels resulting from the use of STR-TP10 were comparable to those obtained with Lipofectamine 2000, and negligible cytotoxicity was observed. Along the same lines, Lehto et al.[23] reported the applicability of an STR-$(RxR)_4$ peptide (x = 6-aminohexanoic acid) for splice correction. The above results suggest that stearylation improved the transfection efficiency of CPPs other than oligoarginines.

Chloroquine is a lysosomotropic agent that induces osmotic swelling and so promotes the disruption of endosomal compartments. Significantly improved splice correction was observed after the treatment of cells with an STR-TP10/2′-OMe ON complex in the presence of chloroquine.[22] However, in terms of future in vivo applications, this strategy is impractical because chloroquine must be taken up with the STR-TP10/NAP complex to obtain improved endosomal escape. PepFect6 (PF6) was thus developed by Langel and his fellow researchers as a novel carrier peptide.[24] PF6 shares the same peptide sequence as STR-TP10 (PepFect 3, PF3); however, four trifluoromethylquinoline moieties are covalently connected to a lysine side chain to improve endosomal escape of the peptide. Even bearing these trifluoromethylquinoline moieties, PF6 formed nanoparticles with siRNA 70−100 nm in diameter in water and 125−200 nm in serum-containing media. As determined by a liposome leakage assay, the PF6/siRNA complex showed an increased membrane destabilization effect at pH 5.5 (endosomal pH) than at pH 7.4, suggesting pH-dependent endosomolytic properties. Effective siRNA delivery was attained into human umbilical vein endothelial cells and Jurkat cells in vitro and in vivo. Intravenous administration of the PF6 complex with siRNA targeting the endogenous hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene resulted in significant reductions in HPRT1 mRNA levels (knockdown efficacy: 50−70%) in the kidneys, lungs, and liver with no significant cytotoxicity.[24] PepFect 14 (PF14) is a novel stearylated peptide that is a modified version of STR-TP10. It was modified with ornithines instead of lysines (in STR-TP10) as a source of positive charge. PepFect 14 can produce solid nanocomplex formulations and yield remarkable splice-correction activity comparable to that from the freshly prepared nanocomplexes in solution.[25] Another

interesting stearylated CPP (NickFect) was reported by Oskolkov et al.,[26] a STR-TP10 analogue having phosphorylated Tyr side chain, yielding an efficient ON delivery vector.

The strategy of attaching a stearyl moiety to form a core–shell structure was also employed by Okada and co-workers.[27,28] A stearylated peptide comprised of cysteine, histidine, and arginine (STR-$CH_2R_4H_2C$) was intended to compact the siRNA with the help of the stearyl moiety and additional disulfide cross-linking between cysteines. Histidine residues may also contribute to endosomal membrane disruption due to a proton sponging effect.[27] STR-$CH_2R_4H_2C$ showed a higher transfection efficacy than Lipofectamine, even in the presence of serum. STR-$CH_2R_4H_2C$ was also employed for in vivo siRNA delivery; the injection of a VEGF-targeting siRNA complexed with this peptide in tumor-bearing mice produced a strong antitumor effect.[28]

Other promising nucleic acid delivery approaches (other than stearylated CPPs) include the 20-residue amphipathic peptide CADY (Ac-GLWRALWRLLRSLWRLLWRA-cysteamide) reported by Divita and co-workers.[29,30] The self-assembly of CADY and siRNA formed a "raspberry"-like particle capable of efficient siRNA delivery in vitro and in vivo. Eguchi et al.[31] reported siRNA delivery using a fusion protein consisting of TAT and a double-stranded RNA-binding domain (Tat-DRBD).

3. NAP Delivery Using CPP-Modified MEND

Stearylated CPPs provide novel methods for the intracellular delivery of NAPs. These approaches have the advantage of easy and simple methods of formulation. On the other hand, various polymer-modified liposomes and macromolecular carriers, including those based on polyethylenimine, chitosan, dendrimers, and nanogels, have been investigated for their potential as nonviral vectors to attain safe and efficient delivery of NAPs into target cells. Of these, polymer-modified liposomes are attractive for NAP delivery and are capable of encapsulating NAPs without the need to alter their chemical structures; moreover, the feasibility of design via surface modification or arrangement of the lipid composition of liposomes gives them another advantage as an NAP delivery vector.[14,15,32] The addition of other targeting moieties (e.g., homing or fusogenic peptides) will enhance the delivery efficiency and biological effects. Biodegradability may reduce the peptides' toxicity and antigenicity. Therefore, ongoing efforts seek to accelerate the cellular uptake of CPP-employing liposomes to obtain greater biological responses.

The first report of CPP-modified liposomes was made by Torchilin and co-workers.[33,34] TAT-modified liposomes using a poly(ethylene glycol) (MW 3000) linker were required to connect the TAT peptide and phosphatidylethanolamine (PE) in order to successfully implant the TAT segment within the liposomal membrane to attain maximum internalization. TAT-liposomes containing a small quantity of a cationic lipid formed noncovalent complexes with DNA and were used for transfection both in vitro and in vivo.

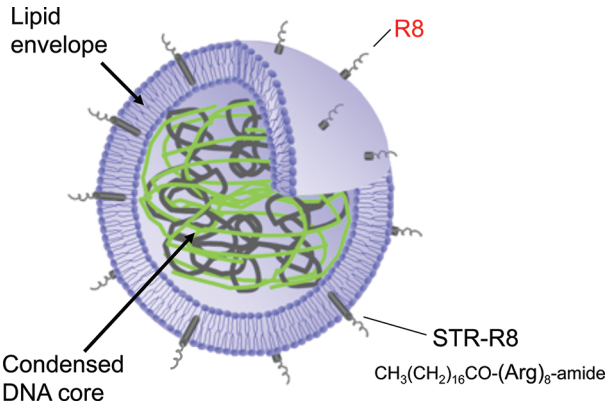


FIGURE 2. Schematic representation of R8-MEND.

Khalil and co-workers[35,36] developed an R8-modified liposome encapsulating pDNA composed of a condensed pDNA core with cationic molecules, including poly-L-lysine, STR-R8, and protamine, coated with a lipid membrane. The modification of liposomes with R8 was easily achieved by the addition of STR-R8 to the liposomal solution, leading to insertion of the stearyl moiety into liposomal membranes.[35] This method of liposome modification (MEND) is compatible with other conventional surface modification methods and thus allows flexible and variable liposome design or "programmed packaging" (Figure 2).[32,35,36] The modification of liposomes with 5 mol % STR-R8 produced the greatest internalization efficacy, attaining a transfection activity as high as that of adenovirus in dividing cells with low cytotoxicity.[36] The in vivo potential of R8-MEND was demonstrated by the topical application of R8-MEND particles containing constitutively active bone morphogenetic protein type IA receptor, which yielded a significant effect on hair growth.[36] R8-MEND is also capable of the efficient delivery of siRNA into cells. Ex vivo siRNA delivery to primary mouse bone marrow-derived dendritic cells, with the eventual aim of developing a cancer vaccine, has been reported.[37,38] The development of ever more sophisticated MEND concepts continues. This includes T-MEND,[39] which has a tetra-lamellar structure (i.e., two nuclear membrane-fusogenic inner envelopes and two endosome-fusogenic outer envelopes) to facilitate stepwise fusion with endosomal and nuclear membranes and efficient DNA delivery

to the nucleus. GALA/PPD-MEND(PPD = poly(ethylene glycol)−peptide−dioleoylphosphatidyl ethanolamine) was developed for cancer targeting, the internalization of which is triggered by matrix metalloproteinase.[40]

4. Internalization Mechanisms of CPPs and Intracellular NAP Delivery

Despite the large number of reports of the successful intracellular delivery of bioactive molecules in vitro and in vivo using CPPs, the precise mechanisms of their internalization remain controversial. One of the reasons for the difficulty in elucidating these mechanisms may be that in many cases CPPs can interact with multiple cell surface molecules, including membrane lipids and membrane-associated proteoglycans. Therefore, it is likely that CPPs can be taken up by cells via multiple pathways, including direct penetration of the plasma membrane and endocytic uptake mediated by clathrin, caveolae, and/or other molecules, depending on the nature of the peptide/cell interaction.[3,13,41,42] Differences in the physicochemical properties of CPPs and cargo molecules also affect their fate after uptake. Elucidation of the cellular uptake mechanisms of CPPs should improve the delivery efficacy of NAPs using CPPs. The most well-studied mechanisms of internalization may be that of arginine-rich CPPs, including TAT and oligoarginine peptides, which share similar methods of internalization.

Whereas both arginine and lysine are representative basic amino acids, oligoarginines generally have a higher internalization efficiency compared with oligolysines possessing the same number of residues. Arginine is a stronger base (pK_a of the guanidino function: ~12.5) than lysine (pK_a of the ε-amino function: ~10.5), while many other cationic nucleic acid carriers are even weaker bases that utilize the proton sponge effect to promote endosomal escape.[43] In addition, the guanidino function may form two hydrogen bonds with phosphate, sulfate, and carboxylate in cell-membrane-associated molecules (e.g., lipids and glycosaminoglycans) (Figure 3), whereas lysine can form only one hydrogen bond with these groups. Whether the difference in basicity or the ability to form hydrogen bonds is a stronger determinant of cellular uptake efficacy is debatable. Rothbard et al.[44] showed the importance of hydrogen bond formation in the superiority of arginine over lysine in an elegant study using N^G-methyl arginine; methylation of the guanidine function should increase the basicity and diminish the hydrogen bond formation capacity. The cellular uptake of octamethylarginine was only ~20% that of nonmethylated R8, indicating the importance of hydrogen bond formation in the cellular uptake of arginine-rich CPPs. Sakai et al.[45] and Rothbard et al.[44] also suggested the possibility of hydrogen bond formation by

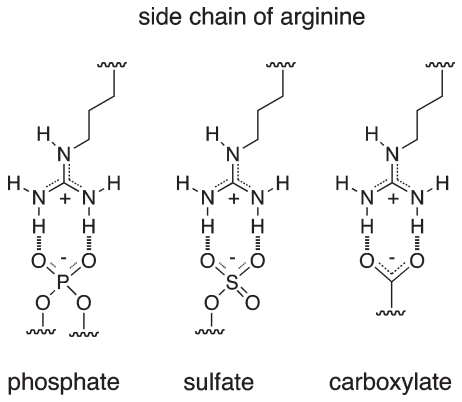


FIGURE 3. Possible mode of hydrogen bond formation between arginine and membrane-associated functional groups.

oligoarginine peptides containing hydrophobic counteranions such as phosphatidylglycerol and lauric acid; these may also assist in the translocation of oligoarginines through membranes. Similarly, enhanced internalization of arginine-rich CPPs and complex formation with splice-switching ONs in the presence of a hydrophobic counteranion, pyrenebutyrate, has been reported.[46−48] These results suggest that arginine promotes cellular uptake more efficiently than lysine due to its hydrogen bond formation capacity.

While the feasibility of direct membrane penetration by oligoarginine CPPs and conjugates has been suggested, especially when attached to relatively small cargo molecules (MW $<\sim$2000),[4,42,49] endocytosis is considered to be the dominant cellular uptake pathway for intracellular NAP delivery using CPPs. For the cellular uptake of arginine-rich CPPs and their NAP conjugates, membrane-associated proteoglycans, including heparan or chondroitin sulfate proteoglycans, play a role in the accumulation of these molecules on cell surfaces via an interaction between the guanidino function of arginine and sulfates in the proteoglycans.[50,51] Nakase et al.[52] and others[53] have proposed that macropinocytosis, accompanied by actin reorganization, plasma membrane ruffling, and the stimulated engulfment of large volumes of extracellular fluid, serves as a major pathway of endocytosis for arginine-rich CPPs (Figure 4). Macropinocytosis does not usually operate in cells; it is activated only when specific stimuli (e.g., growth factors or viruses) are applied.[54] Nakase and co-workers[51,55] suggested that membrane-associated proteoglycans serve as the primary receptor to induce macropinocytosis. Therefore, the interaction of arginine-rich CPPs and their cargos with membrane-associated proteoglycans leads not only to their accumulation on the cell surface but also to the induction of macropinocytosis to accelerate their uptake. The diameters of these macropinosomes are much greater ($>\sim$1 μm) than those for clathrin- or

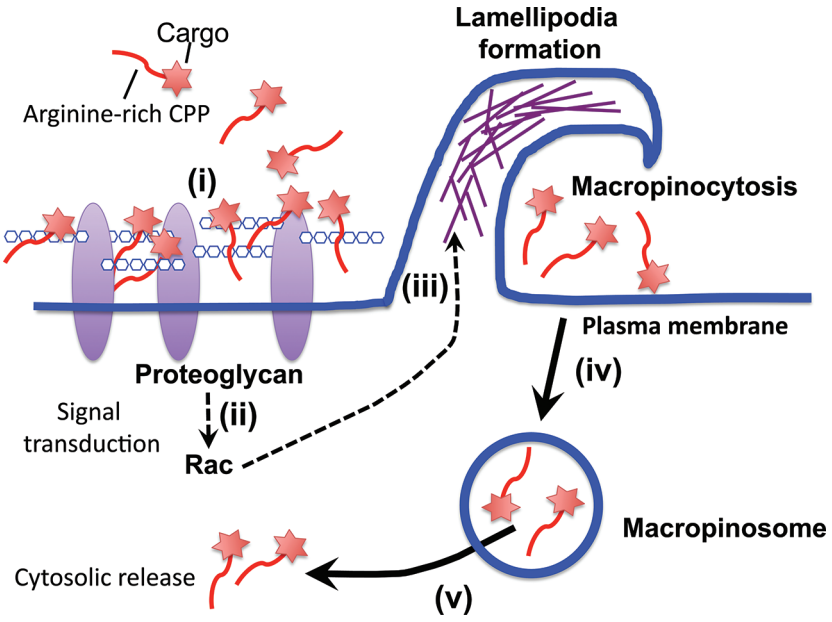


FIGURE 4. Proposed methods for the intracellular delivery of bioactive cargos using arginine-rich CPPs. (i) The interaction of arginine-rich CPPs with membrane-associated proteoglycans leads to (ii) Rac activation, and (iii) actin reorganization, lamellipodia formation, and the induction of macropinocytosis. (iv) Cellular uptake and (v) cytosolic release of the cargos from macropinosomes lead to exertion of their bioactivity.

caveolae-mediated endocytosis (~120 and 80 nm, respectively),[56] which may allow easier cellular uptake of nanoparticles, including R8-MEND. This may be another advantage of arginine-rich CPPs for intracellular NAP delivery.

Studies of the internalization mechanism of arginine-rich peptides have implications for the method of uptake of R8-modified MEND. R8-MEND also binds electrostatically to negatively charged proteoglycans on the cell surface. The methods of cellular uptake of R8-MEND vary depending on the density of arginine. R8-MEND containing 5 mol % STR-R8 liposomal lipids implanted on the surface was taken up by cells via macropinocytosis, whereas that bearing 0.5 mol % STR-R8 was taken up predominantly through clathrin-mediated endocytosis.[35] This result is in agreement with the previous finding that increased clustering of arginine on the cell surface facilitated macropinocytosis and stimulated the uptake of arginine-rich peptides.[52,55] It was also found that the macropinocytic uptake of R8-MEND modified with 5 mol % STR-R8 suffered from less lysosomal degradation than uptake modified with 0.5 mol % STR-R8 via clathrin-mediated endocytosis.[35,57] Thus, optimization of the conditions for macropinocytosis should lead to the mechanism-based design of NAP delivery, resulting in enhanced cellular uptake and bioactivity.

5. Conclusions

The use of arginine-rich CPPs has various implications for NAP delivery into cells. Stearylation of these peptides may lead to the formation of self-assembling nanoparticles with NAP-containing core–shell structures and facilitate cellular uptake. As in the case of PF6, modification with accelerating agents for endosomal escape may further improve the transfection efficiency. Stearylated arginine-rich CPPs are not only useful for the compaction of NAPs, they can be easily applied to the surface modification of liposomes with CPPs, as was shown for R8-MEND. Modification of the liposomal surface charge density may alter the method of cellular uptake, again as found for R8-MEND. Since the activation of macropinocytosis stimulates cellular uptake of extracellular fluid, leading to engulfment into a large endosome (macropinosomes), strategies aimed at stimulating macropinocytosis may stimulate cellular uptake of liposomes and other nanoparticles. Therefore, CPPs provide a platform for the design of efficient intracellular NAP delivery systems. A greater understanding of cellular uptake mechanisms should also contribute to further improvements in delivery systems, and the concepts of core–shell particle formation and MEND may assist in their rational design.

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FOOTNOTES

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