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Oligoarginine-PEG-lipid particles for gene delivery

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Cell-penetrating peptides (CPPs) are small peptides that can facilitate the uptake of macromolecular drugs, such as proteins or nucleic acids, into mammalian cells. Cytosolic delivery of CPPs could be beneficial to bypass conventional endocytosis in order to avoid degradation in the lysosomes. Oligoarginine conjugates have characteristics similar to CPPs in terms of cell translocation and are used in the intracellular delivery of plasmid DNA. In these cases, oligoarginine length and/or charge are important factors in the cellular uptake of oligoarginine alone. The arginine moiety of oligoarginine-modified particles may also be a decisive factor for vectors to deliver plasmid DNA. Oligoarginine-PEG-lipids can form self-assembled particles and modify the surface of lipid- and polymer-based particles. This review focuses on the influence of: i) oligoarginine-modified particles such as micelles, liposomes and polymer-based particles; ii) the morphology of oligoarginine-PEG-lipid complexed with plasmid DNA by decreasing the charge ratio; and iii) the oligoarginine length in the complex on its cellular uptake, transfection efficiency and uptake mechanism. The oligoarginine length of oligoarginine-modified particle complexed with plasmid DNA governs the cellular uptake pathway that determines the destiny of intracellular trafficking and finally transfection efficiency. The new aspects of surface-functionalized particle vectors with oligoarginine are discussed.

Keywords: cell-penetrating peptides, endocytosis, gene delivery, liposome, micelle, oligoarginine, particle vector, plasmid DNA, polyplex

Expert Opin. Drug Deliv. (2009) 6(10):1065-1077

1. Introduction

For gene delivery, viral vectors provide very high transfection efficiency, but their safety is a great concern because of their immunogenicity and acute toxicity [1,2]. For the future development of gene therapy, a safe and highly effective non-viral vector is indispensable. For this purpose, non-viral vectors such as cationic liposomes and polymers have been developed [1,3]; however, their low transfection efficiency, compared with viral vectors, is considered to be a major limitation in their application to gene therapy. The major cellular uptake mechanism of non-viral vectors is classical clathrin-dependent endocytosis [4,5]. Transfection efficiency may be limited by the ability of cationic lipids or polymers to form a complex with plasmid DNA (DNA), to enter the cell, escape the endosome, release the plasmid DNA (DNA) into the cytoplasm, or by the entry of DNA into the nucleus. To escape the endosome, new and more efficient synthetic vectors, hopefully with a different cell internalization mechanism from classical clathrin-dependent endocytosis, are desired.

One approach to circumvent the problem is to incorporate short peptides derived from cell-penetrating peptides (CPPs), such as HIV-1 TAT fragments. CPPs are peptides < 30 amino acid residues in length that have the ability to cross the plasma membrane [6-11] and can deliver a wide variety of cargos, including

10.1517/17425240903156366 © 2009 Informa UK Ltd ISSN 1742-5247

Table 1. Oligoarginine-modified derivative vectors.

Oligoarginine-modified derivative vectors	Ref.
DNA	
Argininedodecylester	[36]
Stearyl-R8	[28]
Arginine-based cationic lipid	[37]
Oligoarginine (R4-R10) -PEG-lipid	[38,39]
Arginine-cholesterol-liposome	[40]
Bile acid-oligoarginine (R3, R4, R6) conjugate	[41]
Generations 2, 3 and 4 arginine modified dendrimer	[42]
Arginine-conjugated polypropyleneimine dendrimer	[43]
Arginine-grafted bioreducible poly(disulfide amine)	[44]
Arginine-chitosan	[45]
R9-PEG-polyethyleneimine	[46]
siRNA	
R9-conjugated CD7-specific single-chain antibody	[31]
R9-conjugated peptide derived from rabies virus glycoprotein	[32]
Stearyl-R8	[30]
Cholesterol-R9	[33]
Stearyl-R8-modified liposome	[34]
R8-PEG-liposome	[35]

peptides, proteins and nucleic acids [12-14]. As CPPs can deliver conjugated molecules, such as proteins and genes, into cells [15,16], they are frequently used as conjugates with cargo molecules. Thus, these CPP carriers have been crosslinked to the target protein or gene. Oligoarginine conjugates have been demonstrated to have characteristics similar to CPPs in terms of cell translocation [10,17,18]. TAT-PEG-polyethyleneimine/DNA complex [19], ternary (DNA/TAT)/polyethyleneimine complex [20], (DNA/TAT)/cationic lipid [21] and TAT/liposome/DNA [22] can all facilitate gene transfer. As CPP-modified nanoparticles [23] and liposomes [24-26] can be taken up into cells, liposomes modified with TAT were used for the intracellular delivery of DNA and drugs entrapped in liposomes [27]. The influence of oligoarginine length on uptake of oligoarginines alone and transfection efficiency has been reported [10,17,18,28], but that of oligoarginine-modified particles has not been reported. Furthermore, although as a particle in many cases liposomes have been used, other particles such as nanoparticles and micelles, and so on, have not been studied in detail. This review focuses on the influence of: i) oligoarginine-modified particles such as micelles, liposomes and polymer-based particles; ii) the morphological changes in oligoarginine-PEG-lipid complexed with DNA resulting from decreases in the charge ratio; and iii) the oligoarginine length of the complex on its cellular uptake, transfection efficiency and uptake mechanism. The oligoarginine length of oligoarginine-modified particles complexed with DNA governs the cellular uptake pathway that determines the destiny of intracellular trafficking and finally transfection efficiency rather than the morphology of the complex, such as particle size and zeta-potential.

2. Relationship of oligoarginine length of oligoarginine-PEG-lipid particles with transfection efficiency

A peptide consisting of oligoarginine has been shown to be translocated through the cell membrane as efficiently as other CPPs [10,17,18]. Their oligoarginine length [10,17,18] and the hydrophobic moiety such as lipids of oligoarginine conjugates [28] were important factors for the uptake by and transfection of cells. Mitchell *et al.* reported that 15 arginine residues were internalized significantly more effectively than 20 arginine residues [17]. Wender *et al.* reported that nine arginine residues, the maximum number used in their experiment, were superior to shorter oligomers in terms of cellular uptake as determined by flow cytometry [18]. They also demonstrated that the presence of at least six arginine residues is important for cellular uptake [18]. Using oligomers composed of 4 – 16 arginine residues, Futaki *et al.* demonstrated that there was an optimal number of arginine residues (R8) for cellular internalization by microscopic observation using fixed cells [10]. Later, using living cells, the same group [29] reported that the cellular uptake efficiency of R18 peptide was higher than with R8 peptide. They also reported that stearylation of R8 at the N terminus (stearyl-R8) improved the transfection efficiency compared with R8, giving the highest transfection efficiency from stearyl-R4 to stearyl-R16 [28]. Stearyl-R8 and lipid-based (artificial viral envelope) delivery methods were applied for the transfection of siRNAs into rat primary neurons [30]. R9-conjugated CD7-specific single-chain antibody [31,32], R9-conjugated peptide derived from rabies virus glycoprotein (RVG) [31,32], R9-conjugated cholesterol [33] and R8-modified liposomes [34,35] were also used for siRNA delivery. The oligoarginine-conjugated lipid [28,36-40], bile acid [41], dendrimer [42,43] and polymer [44-46] derivative vectors for complexes with DNA and siRNA are listed in **Table 1**.

A study by the current authors designed new oligoarginine-modified lipids, 3,5-bis(dodecyloxy)benzamide (BDB) with and without a PEG spacer, (Arg)n-BDB (Rn) or (Arg)n-PEG-BDB (RnB) of various oligoarginine lengths (n = 4, 6, 8, 10) as a self-assembly system (**Figure 1**) [38]. BDB is an artificial lipid. RnB (R4B – R10B) could spontaneously form micelles in water because of the characteristic function of the PEG-lipid part to form micelles (**Figure 1B**). The critical micelle concentration (CMC) value of R4B and R10B was 5.7 μM (17.6 μg/ml) and 20.9 μM (83.6 μg/ml), respectively [39]. When mixed with DNA, RnB micelles could form a complex with DNA (RnB-micelle complex). Abbreviations used for specific oligoarginine-PEG-lipid complexes with DNA are

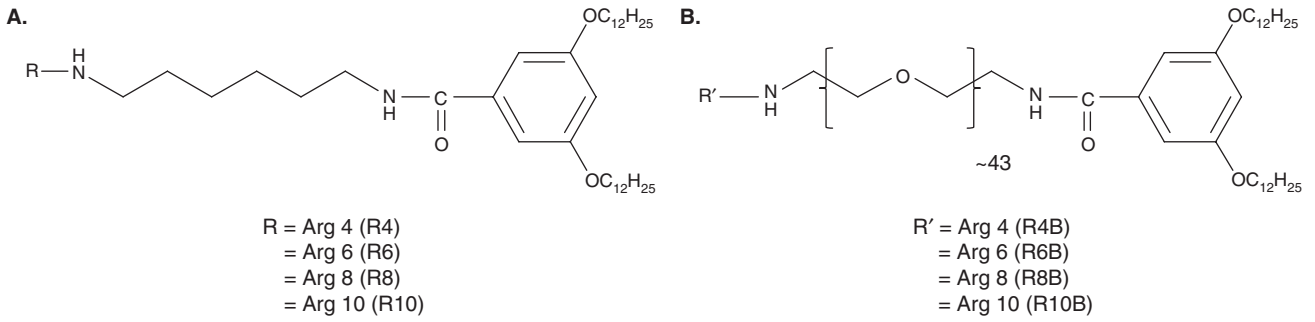

Figure 1. Chemical structures of (A) (Arg)n-BDBs and (B) (Arg)n-PEG-BDBs.

listed in **Table 2**. The RnB concentration is an important factor in obtaining high transfection efficiency by luciferase assay in HeLa cells. Micelles formed of longer RnBs at a concentration of 100 μg/ml showed stronger luciferase activity, that is, R10B micelles showed the highest level of activity among the RnB derivative micelle vectors at mixing ratio ± of RnB to DNA of 5.5-4.2 (positive charge of RnB was considered as the same (+1), not depending on Rn residues, mixing ratio ± x n = the lipid nitrogen/DNA phosphate (N/P ratio) [38]. Serum tended to decrease the gene transfection efficiency of R4B up to R8B micelles, whereas serum did not significantly decrease that of R10B micelle [38]. R12B-micelle complex showed higher cellular uptake and slightly higher transfection efficiency than R10B-micelle complex after incubation only with serum because serum tended to decrease higher cytotoxicity of R12B-micelle complex than that of R10B-micelle complex, owing to higher cationic charge (unpublished data). A component of serum may neutralize the charge of RnB. Interestingly, two quite different designs of oligoarginine lipids, R10B (25 μM) [38] and stearyl-R8 (30 μM) [28], showed similar transfection efficiencies to a commercial gene transfection reagent, Lipofectamine™ 2000 (Invitrogen, CA, USA). The transfection efficiency by RnB-micelles is likely to be influenced more by the length of oligoarginine than by overall structural features such as the anchor lipids, spacer groups, or the direction of oligoarginine relative to the lipid portion, and so on [38]. Thus, R10B is potentially a simple and useful tool, because micelle vectors are easy to prepare by just mixing aqueous solutions without the need to manufacture particles previously.

Torchilin *et al.* first reported the use of CPP-modified liposomes for gene delivery [26]. They demonstrated that liposomes can be delivered into cells by attachment of TAT in the presence of high-molecular-mass PEG molecules to the liposome surface. Liposomes modified with Rn or RnB (Rn-liposome, RnB-liposome) have also been prepared [38]. The surface of liposomes of ~ 200 nm in diameter seemed to be saturated by the modification of 5 mol% R10 because there was no further increase of the zeta-potential of the prepared liposomes [47]. Rn- and RnB-liposomes containing 5 mol% of Rn or RnB were used in the experiments, because highly positively charged carriers are desirable to form complexes with DNA and to deliver it into cells. The zeta-potentials of Rn-liposomes increased as the oligoarginine length increased from R4 to R10 [47]. The pH dependency of the zeta-potential of R10-liposomes (pI > 10) was not influenced significantly after incubation with a highly negative protein, bovine serum albumin, using measurement by the pH titration method [47]. The zeta-potential of R10-liposomes may be scarcely changed by the adsorption of protein in incubation medium with serum. The change in cellular uptake of Rn- and RnB-liposomes can be interpreted depending on the oligoarginine length. Longer oligoarginine showed stronger luciferase activity, and R10B had the highest level of activity among the series of liposomes tested. The optimal number of arginine residues for transfection proved to be 10 or more. PEG-spacer was effective for transfection because R10B-liposomes showed about 50-fold higher transfection efficiency than R10-liposomes [48].

3. Effect of decaarginine-PEG-lipid concentration to DNA on complex morphology and transfection efficiency

Surfactant and lipid-based micelle vectors for the transfection of DNA are summarized in **Table 3**. Surfactants [49,50], cationic detergent polymerized by the formation of intermolecular disulfide bonds within DNA [51] and dimerizable cationic thiol detergents [52,53] have been used. The effect of cationic surfactant concentration on the morphology of DNA complex and its transfection efficiency has been reported. Pitard *et al.* [54] reported that the supramolecular structure of lipopolyamine micelle complex showed high transfection efficiency. Inverted hexagonally packed cylinders of cationic lipid micelle complex were suitable for endocytosis uptake or vesicle fusion [55].

A previous study revealed that R10B showed higher transfection efficiency at a concentration below the CMC rather than above the CMC [39]. Micelle formation of R10B is not necessary for high transfection efficiency [48]. At a concentration above the CMC, the R10B-micelle complex (N/P ratio = 42.5/1, mixing ratio ± of RnB to DNA of 4.2) was ~ 1.5 μm in diameter and 42.7 mV for zeta-potential [39].

Table 2. Abbreviations of oligoarginine-PEG-lipid complexed with DNA.

Lipid-particle, complex	Abbreviation	
Arg(n)-PEG-BDB	RnB	Concentration below CMC
	RnB-micelle	Concentration above CMC
Arg(n)-liposome	Rn-liposome	
Arg(n)B-liposome	RnB-liposome	
Protamine/DNA	Polyplex	
Arg(n)B/DNA	RnB-lipoplex	
	RnB-micelle complex	Concentration above CMC
Arg(n)B/protamine/DNA	RnB-polyplex	
Arg(n)-liposome/DNA	Rn-lipoplex	
Arg(n)B-liposome/DNA	RnB-lipoplex	

Table 3. Surfactant and lipid-based micelle vectors for transfection of DNA.

Micelle vector	Ref.
N-*N*-dioleoyl-*N, N*-dimethylammonium chloride (DODAC)	[49]
Cetyltrimethylammonium bromide	[50]
Dimerizable cationic detergent, C12-16COrn	[52]
Cationic detergent, tetradecylamide of ormithylcysteine (C_{12} CCP)n	[51]
Poly[[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium iodide] ethyl phosphate] (PCEP)	[94]
Lipopolyamine (RPR120535)	[4,54,95]
Cationic lipid (TRX)	[55]
Oligoarginine-PEG-lipid	[38,39]
Chitosan oligosaccharide-stearic acid	[96]
Dimerizable triazine-based cationic lipid	[53]

At a concentration below the CMC, the R10B-lipoplex (N/P ratio = 8.5/1) was ~ 200 nm in diameter and with a zeta-potential of 27.1 mV, which decreased owing to the increase in negatively charged DNA.

The nanostructure of R10B-complexes under various lipid concentrations was observed by phase contrast cryo-transmission electron microscopy (TEM) at two higher concentrations of R10B at the same N/P ratio as the transfection experiments (**Figure 2**). At an R10B concentration above the CMC, micellar structures with several nanaometer sizes were observed. With R10B-lipoplex at a lower N/P ratio, a net-like structure was observed, in which DNA was involved (**Figure 2A**). These net-like structures may contribute to high transfection efficiency because R10B showed higher transfection efficiency at a concentration below the CMC rather than above the CMC [48]. In the R10B-micelle complex, heterogeneous nanostructures were observed (**Figure 2B**). Other than net-like structures, large fibrous nanostructures were visible.

An increase in lipid concentration to DNA tended to convert the net-like structures into a large fibrous structure. As the lipid part of RnB, BDB, became a fibrous structure with the increase in lipid concentration [56], DNA may induce the fibrous nanostructure by partial neutralization of R10B at higher concentration of R10B. DNA may modulate the net-like structure and fibrous nanostructure of R10B. It was confirmed by fluorescence intensity distribution analysis (FIDA) that the net-like structure showed stronger interaction between fluorescence-labeled DNA and R10B than the fibrous nanostructure.

4. Relationship of oligoarginine length, decaarginine-PEG-lipid concentration and PEG-spacer to cellular uptake

The quantitative uptake of free CPP or CPP coupled to cargo can differ [57,58]. Similar to oligoarginine peptides, the cellular uptake of RnB-micelle complex, which was fluorescence labeled at the lipid terminus, was proportional to oligoarginine length up to 3 h after incubation as well as transfection efficiency [38]. Considering the effect of oligoarginine length of the RnB-micelle complex on cellular uptake to transfection efficiency, the arginine residues may be partly used for translocation through the plasma membrane and partly for the formation of complex with DNA. The longer Rn could be needed for the intracellular delivery of RnB-micelle complex than for the uptake of RnB alone. In this respect, RnB with > 10 arginine residues could be more effective for transfection if cytotoxicity is not increased, because the cytotoxicity of RnB-micelle complex increased with the increase in oligoarginine length (unpublished data).

The cellular uptake of R10B-lipoplex was similar below and above the CMC of R10B concentration, although the transfection efficiency below the CMC was significantly higher than that above the CMC [59]. The zeta-potential and size of lipoplex were not determinant factors for transfection, possibly owing to a change of morphology due to the R10B concentration. R10B-lipoplex showed ~ 1000-fold higher transfection efficiency to R4B-lipoplex in spite of similar cellular uptake [59].

Unlike in the micelle complex, the effect of oligoarginine length of Rn- and RnB-liposomes on cellular uptake was in inverse proportion to the transfection efficiency [38]. Longer Rn length showed lower uptake and higher transfection efficiency. This cellular uptake dependency of oligoarginine length was confirmed using both fluorescently labeled oligodeoxynucleotide (ODN) and DNA in lipoplexes. The

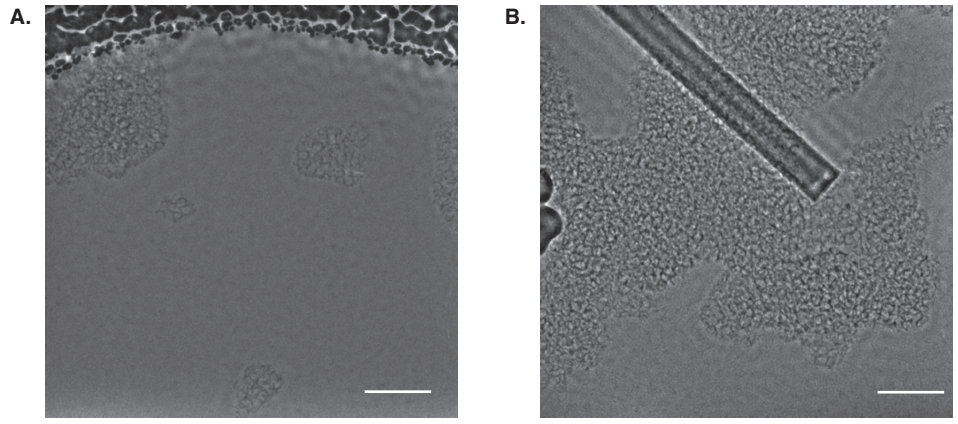

Figure 2. Phase contrast cryo-TEM analysis of the structure of R10B-lipoplexes. A. N/P ratio = 8.5/1, corresponding to 5 μM R10B/2 μg DNA. **B.** N/P ratio = 42.5/1, corresponding to 25 μM R10B/2 μg DNA. Scale bar = 100 nm.

liposome was internalized as intact vehicle, which was confirmed by the measurement of fluorescence uptake in the cells using it as a marker of the aqueous phase in liposomes, and also using fluorescently labeled liposomes and ODN [47]. This opposite dependency of oligoarginine length in micelles and liposomes on their uptake may be caused by the lipophilic nature of liposomes. A hydrophobic lipid structure with shorter Rn may affect the release of DNA in the cytoplasm.

The discrepancy between transfection efficiency and cellular uptake indicated that intracellular trafficking was one of the limiting stages for transfection efficiency, but not for the uptake process. The endosomolytic properties of RnB-lipoplex were investigated using chloroquine, which is known to inhibit intracellular degradation by lysosomal enzymes because it is an endosome-disrupting agent [60]. The presence of chloroquine significantly improved the transfection efficiency of R4B-lipoplex more than that of R10B-lipoplex. R4- and R4B-liposomes interacted with DNA more strongly than with R10- and R10B-liposomes, which was detected by electrophoresis [59]; therefore, these liposomes could not release DNA into the cytoplasm. Collectively, it is reasonable to suggest that the cellular uptake mechanism of oligoarginine-modified particles may differ according to the oligoarginine length.

Surface functionalization through the PEG spacer was mostly reported for targeted drug delivery [61]. The PEG spacer could affect transfection by allowing greater oligoarginine flexibility resulting in more efficient interaction with the cell membrane. Torchilin *et al.* [26] reported that cellular uptake and the transfection efficiency of TAT peptide attached to the liposome through the PEG spacer were higher than those attached directly to the liposome. R10B-lipoplex showed lower cellular uptake but significantly higher transfection efficiency than R10-lipoplex at the same particle size and N/P ratio. PEG spacer in R10B-liposomes may be more effective for intracellular trafficking, such as release of DNA in cytoplasm, rather than cellular uptake by oligoarginine [48].

5. Improvement of transfection efficiency

5.1 Oligoarginine coating polyplex

Polyplex is a nanometric particle composed of cationic polymer and DNA that can be coated with lipid on the surface to increase transfection efficiency. Protamine is arginine-rich protein, which has been reported to act as a nuclear localization signal [62] and has a possibility to facilitate the intracellular release of nucleic acid [63]. Accordingly, DNA partially neutralized with protamine may promote the release of DNA from endosomes into the cytoplasm and the penetration of DNA into the nucleus. Negatively charged protamine/DNA complex (polyplex) was prepared at a charge ratio (N/P) of 0.3 and then the polyplex was coated with RnB at various mixing ratios ± of RnB to DNA [64]. RnB is water-soluble and may coat polyplex, which confirmed that the zeta-potential of RnB-polyplex was changed from negatively charged polyplex alone (-30 mV) to a decreased negative charge or positive charge with the increase in RnB. The particle size of RnB-polyplex was ~ 150 – 220 nm, and its zeta-potential increased in parallel with the increasing mixing ratio ± of RnB to DNA.

RnB-polyplex increased the transfection efficiency with an increase in the mixing ratio ± and showed higher transfection efficiency than the corresponding RnB-lipoplex in HeLa cells (**Figure 3A**). R10B-polyplex at a mixing ratio ± of R10B to DNA of 1 was the highest among RnB-lipolplex and RnB-polyplexes. Unlike the large differences in transfection between R4B and R10B, similar uptake amounts were observed in both polyplexes. As, like other particle vectors, the high uptake of R4- and R4B-polyplex did not reflect their transfection

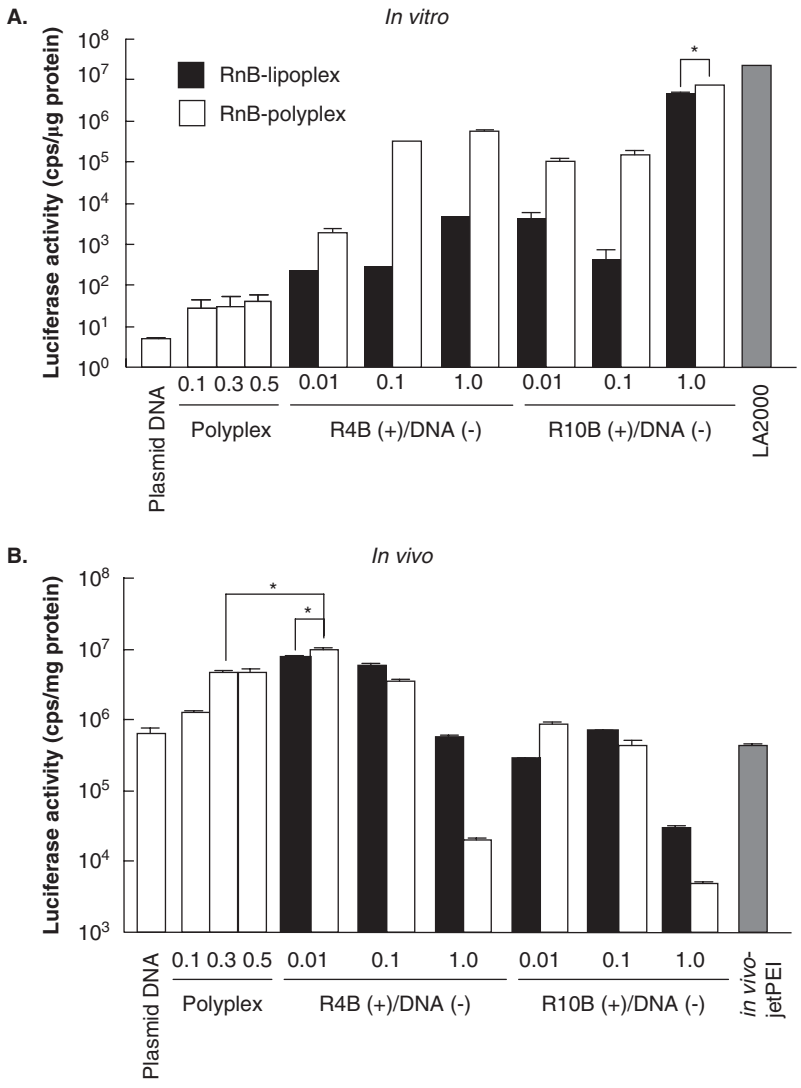

Figure 3. Effect of mixing ratio ± of protamine to DNA for polyplex or RnB to DNA on luciferase activity in HeLa cells (A) and in tumors (B). The N/P ratio of polyplex in RnB polyplex was 0.3. **A.** After 3 h incubation in serum-free medium, cells were incubated further for 21 h at 37°C in medium containing 10% FBS. **B.** Luciferase activity in tumors 24 h post-intratumoral injection of vector/DNA complex into mice at a dose of 10 μg DNA per tumor. Each bar represents the mean ± s.d. of three experiments.
*$p < 0.05$.
Figure adapted from [64].

activity, R4B could not release DNA from the complexes better than R10B even when the negative charge of DNA was partially decreased by protamine.

5.2 Endosomal escape treatment

Ca^{2+} is known to facilitate the transfer of large molecules from the endosome compartment to the cytoplasm [65]. Ca^{2+} was added to the medium after the formation of polyplex because it was reported that this procedure enhanced transfection [66]. A previous study reported that to release DNA from the complexes, the complexes were prepared in the presence of Ca^{2+}, and Ca^{2+} enhanced R4B-polyplex-mediated gene transfer but not R10B-polyplex-mediated transfer [59]. The presence of Ca^{2+} resulted in a maximum increase in R4B-polyplex transfection potency of about 70-fold, similar to Lipofectamine 2000. The addition of Ca^{2+} enhanced the transfection of RnB-lipoplex and RnB-polyplex without increasing its cellular uptake. Ca^{2+} may stimulate the release of DNA from endosomal/lysosomal compartments. It has been reported that efficient transfection of cationic liposomes in the presence of Ca^{2+} is observed at lower cationic lipid-to-DNA charge ratios than in the absence of Ca^{2+} [66]. When lower cationic lipid ratios were considered with R4B, the results corresponded with this report. Ca^{2+} might substitute

to some extent for R4B in the complex and interact directly with DNA. R10B had greater cytotoxicity than R4B, which may be caused by the longer oligoarginine chains. Therefore, R10B-polyplex could not gain an advantage from Ca^{2+} because Ca^{2+} also induced cytotoxicity.

It is known that the role of dioleoyl phosphatidylethanolamine (DOPE) is to facilitate membrane fusion and to aid in destabilization of the plasma membrane or endosome [13,67]. R4- and R4B-liposomes showed the highest level of uptake of ODN but the lowest transfection efficiency among a series of Rn- and RnB-liposomes [48]. R4- and R4B-lipoplexes may not be able to release DNA from the endosome easily. To improve the transfection efficiency of R4- and R4B-lipoplexes, DOPE was added to the composition in Rn- or RnB-liposomes. The presence of DOPE in R10-liposomes increased transfection efficiency much more effectively than that in R4-liposomes at a mixing ratio ± of R10 to DNA of 1. DOPE in RnB-liposomes did not increase either transfection efficiency or cellular uptake compared with RnB-liposomes alone (unpublished data). With the increase of the mixing ratio ± of oligoarginine to DNA, the transfection efficiency of R10-liposomes with DOPE was enhanced further, whereas that of R10B-liposomes with DOPE was not changed or even impaired (unpublished data). Thus, the DOPE addition was shown to be a useful strategy to enhance transfection efficiency, depending on the structure of oligoarginine-modified liposomes.

6. Mechanism of cellular uptake of oligoarginine-PEG-lipid particles

Most of the mechanistic studies of CPP uptake and distribution in cells have been carried out using CPPs alone, and not many reports have looked at the distribution after uptake of CPPs conjugated to biomolecules. The uptake mechanism and cellular distribution of CPPs is not clearly understood. The cellular translocation by CPPs was initially proposed to be an energy-independent process. Most reports have indicated no difference in uptake between 37 and 4°C [10,68]. However, more recent studies have suggested that most of the translocation occurs by means of an energy-dependent pathway and that the translocation of CPPs is reduced by endocytosis inhibitors [68-70]. Several reports indicated that artifacts were caused by cell fixation before confocal laser scanning microscopy of cells incubated with fluorescence-labeled CPPs. Another source of misinterpretation was found in experimental difficulties in distinguishing cell surface-associated CPPs from CPPs internalized into the cytoplasmic compartment [71].

Using the present research techniques, there is a limitation in the examination of uptake pathways because CPPs should be observed in unfixed cells. In addition, the analysis method to examine where the translocation of oligoarginine into cells has been traced using partition properties into membranes, which might be affected when hydrophobic molecules, such as fluorescent markers, are attached to oligoarginine or its modified particles.

Endocytosis represents a variety of mechanisms that fall into two broad categories, phagocytosis and pinocytosis (**Figure 4**) [29,72]. Phagocytosis is typically restricted to specialized mammalian cells, whereas pinocytosis occurs in most cells by means of at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [73]. Interestingly, although most of these pathways lead to delivery of the transported cargo to lysosomes, that is, the degradation compartment in cells, the caveolae- and macropinocytosis-mediated pathway seems to evade this destination [72,74,75]. Caveolae-mediated endocytosis is inhibited by filipin through cholesterol depletion. The chief differences between these endocytosis mechanisms are the size of the vesicles formed on internalization, their regulation and the intracellular trafficking route. This implies that the cargo size might be one decisive parameter for internalization mechanisms and the final intracellular destination.

Macropinocytosis is an actin-dependent endocytic pathway that proceeds via membrane protrusions formed by membrane ruffling that enclose a large volume of extracellular material, followed by pinching off to form intracellular vesicles [76,77]. Macropinocytosis is the most probable endocytosis pathway for larger CPP conjugates, as other types of endocytosis in general are characterized by vesicles < 150 nm. Inhibition of uptake of CPPs, such as TAT, by ion-transport inhibitors, including Na^+/H^+ exchanger inhibitors such as amiloride and its more potent analogue 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), suggests a link between peptide internalization and macropinosomes [76,78]. Macropinocytosis is induced by the phorbol ester 12-myristate 13-acetate (PMA), a potent activator of protein kinase C, which resulted in increased membrane ruffles [77,79,80].

R10 showed similar cellular uptake but higher transfection efficiency than R4 with both complex- and polyplex-type vectors [59]. In the latest reports, the translocation of TAT and R8 peptide are suggested to occur through macropinocytosis [29,78,81], although the uptake pathway still varies with structure of CPP, cargo, cell type, and so on. The first step in transduction is binding to the cell surface, which is likely to comprise an ionic interaction between the positively charged CPPs and negatively charged surface proteoglycans [82,83]. The second step is induction of macropinocytosis, which describes the transduction of arginine residues dependent on guanidinium moiety-rich peptides in the cells. The third step is escape from the macropinosomes.

The internalization mechanism of R10B-micelles and R10-micelle complex has a smaller contribution from energy-independent processes and these complexes following a similar pathway [38]. R10B-micelles alone and the R10B-micelle complex showed similar internalization efficiency at 37°C, lower internalization efficiency by preincubation of cells with a metabolic inhibitor, sodium azide, and stronger inhibition of cellular uptake than transferrin (Tf) or TAT, as endocytosis markers, at low temperature, as analyzed by flow cytometry.

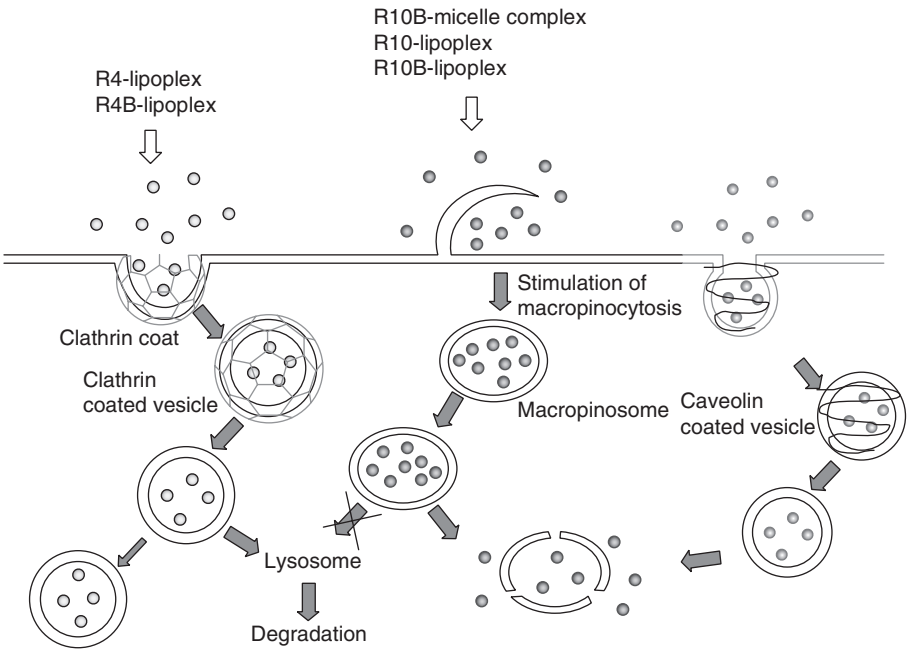

Figure 4. Schematic diagram of endocytosis. Clathrin- and caveolae-mediated endocytosis and macropinocytosis.

Marty *et al.* [25] reported that the uptake of TAT- and Antp-liposomes did not occur through clathrin-mediated endocytosis from colocalized liposomes with Tf. Khalil *et al.* [84] reported that 5 mol% R8-modified liposomes containing condensed DNA showed higher gene expression through macropinocytosis. The cellular uptake of fluorescencently labeled R10-liposomes dropped significantly in the presence of EIPA but increased in the presence of PMA compared with untreated cells. The cellular uptake of R4-liposome was not affected by this treatment [47]. R10-liposomes were taken up mainly through macropinocytosis, but R4-liposomes were predominantly taken up by another route. Unlike with R4- and R4B-lipoplexes, the internalization of R10- and R10B-lipoplexes with fluorescently labeled ODN and DNA was partially inhibited by hypertonic treatment and hardly influenced by caveolae inhibitors such as filipin, but was strongly inhibited by macropinocytosis inhibitors such as EIPA. However, a fraction (~ 30 – 40%) of R10 uptake still occurred independently of inhibition by EIPA [48]. A method of direct observation for multiple mechanisms is needed.

The uptake of R10- and R10B-liposomes did not occur through clathrin-mediated endocytosis because the internalized liposomes were not colocalized with Tf, but those of R4- and R4B-liposomes were colocalized with Tf. R10B-lipoplexes did not show any colozalization of fluorescencely labeled liposomes and DNA using confocal microscopy after 3h incubation, suggesting that release of DNA from R10B-lipoplexes was faster than that from R10-lipoplexes. PEG spacer may promote the escape of DNA from macropinosomes. The results were confirmed further by a colocalization study with fluorescence labeling of both liposomes and DNA where faster release of DNA from R10- and R10B-lipoplexes into cytoplasm was observed than from R4- and R4B-lipoplexes by living confocal microscopy images after a 3 h incubation [48]. Uptake of R10- and R10B-lipoplexes occurs mainly through macropinocytosis and that of R4- and R4B-lipoplexes mainly through clathrin-mediated endocytosis.

R10- and R10B-lipoplexes showed higher transfection efficiency than R4- and R4B-lipoplexes because DNA may be released more easily into the cytoplasm from R10- and R10B-lipoplexes because it is reported that macropinosomes are leaky [85].

With decreasing R10B concentration, R10-lipoplexes are taken up mainly through macropinocytosis, as demonstrated by inhibition of this process by EIPA [39]. This result was in conflict with the reports that the cellular entry of R8/DNA and stearyl-R8/DNA complexes occurs mainly through clathrin-mediated endocytosis [86]. Different nanostructures of complexes utilized the same cellular uptake mechanism but resulted in different transfection efficiencies, because the large fibrous nanostructure might inhibit the release of DNA from macropinosomes into the cytoplasm.

Even with the addition of DOPE, Rn- and RnB-liposomes did not change their uptake pathway. R10- and R10B-liposomes containing DOPE showed uptake by macropinocytosis as the major uptake route, whereas R4- and R4B-liposomes containing DOPE mainly used a different pathway, probably clathrin-mediated endocytosis, because hypertonic treatment inhibited the internalization of R4- and R4B-lipoplexes and the macropinocytosis inhibitor EIPA inhibited that of

R10- and R10B-lipoplexes. DOPE seemed to be more effective in promoting the release DNA from macropinosomes than clathrin-mediated endosomes because R10-liposomes containing DOPE showed increased transfection efficiency more than R4-liposomes containing DOPE.

To investigate the main mechanism for the cellular uptake of decaarginine, both observations by microscopy and quantitative methods for the same samples are needed, in order to monitor intracellular trafficking of CPPs and cargo molecules. The high cationic charges of CPPs induced cytotoxicity, which may be influenced by incubation with or without serum and/or specific cell types, and thus affect the cellular uptake mechanism. More precise results on intracellular trafficking may be obtained using other cell lines.

7. Intratumoral injection of oligoarginine-coated polyplex

In an *in vivo* experiment, R8-modified liposomes with cleavable PEG-lipid could facilitate tumor accumulation and resulted in high gene expression in tumor cells [87]. The topical application of R8-modified liposomes resulted in significant gene expression in hair follicles [88].

A study by the current authors evaluated the transfection efficiency of oligoarginine-coating polyplex (RnB-polyplex) vectors and naked DNA by assaying luciferase activity in HeLa xenograft tumors after intratumoral injection [64]. An *in vivo* transfection experiment showed an inverse correlation with the *in vitro* experiment (**Figure 3B**). R4B-polyplex at a mixing ratio ± of 0.01 had the highest transfection efficiency among the vectors, ~ 2-, 16- and 23-fold compared with the polyplex, R10B-polyplex at a mixing ratio ± of 0.01, and a commercial gene transfection reagent, *in vivo*-jetPEI™ (Polyplus-transfection, France), respectively (**Figure 3B**). The *in vitro* system for evaluating oligoarginine-mediated gene delivery did not reflect the optimal conditions of *in vivo* gene delivery. Although R4B- and R10B-polyplexes at a mixing ratio ± of 1.0 had similar particle sizes and zeta-potentials, their transfection efficiencies were quite different in the tumor, suggesting that oligoarginine length was an important factor for intratumoral gene delivery. Protamine neutralized the charge of DNA. Therefore, R4B may interact with cells as a CPP, and spread widely in tumors. Although the longer oligoarginine of R10B showed high transfection efficiency *in vitro*, excess arginine residues in R10B might inhibit the spread of R10B-polyplex by interaction with tissue proteins. This finding corresponded to the result when direct injection of naked DNA into solid tumor resulted in a high level of transfection, whereas a cationic lipid-based vector inhibited gene expression [89,90]. The difference in gene expression between *in vitro* and *in vivo* might be because of the difference in accumulation of vector/DNA complexes near tumor cells. R4B coated on polyplex worked effectively for intratumoral gene delivery.

In other cell lines, medullary thyroid carcinoma TT and pheochromocytoma PC12, and their xenograft tumors in mice, luciferase expression was confirmed *in vitro* and *in vivo* following intratumoral injection of R4B-polyplex and R10B-micelle complex (unpublished data).

8. Conclusions

Micelle vectors are easy to prepare by just mixing aqueous solutions of oligoarginine lipids and DNA without the need previously to manufacture particles such as liposomes. Micelles changed morphology depending on concentration, and therefore the morphology of micelle complex is easily changed according to the concentration of oligoarginine lipid and its ratio to DNA in the mixture. With a decrease in N/P ratio, DNA polymer dominates the morphology of the complex, which is characterized by the size and self-assembly of the structures, and contributes high transfection efficiency. Liposomes can easily have their functions modified by changing components such as oligoarginine lipid and the PEG spacer, while maintaining their shape as liposomes.

Rich oligoarginine-modified particles, such as micelles, showed higher cellular uptake of DNA molecule with longer oligoarginine length, similar to oligoarginine peptides alone. Poor oligoarginine-modified liposomes showed cellular uptake governed by the lipophilic nature of the liposomes, which did not depend on oligoarginine length, whereas their cellular uptake mechanism was still dependent on oligoarginine length. Decaarginine-PEG-lipid complex induced macropinocytosis as the cellular uptake mechanism of small to large particles, such as micelles and polyplexes to liposomes with ~ 200 nm in diameter, independently of cargo size, change in nanostructure according to concentration, or the presence of PEG spacer and DOPE in the liposome.

PEG spacer in R10-liposomes may be more effective for intracellular trafficking, such as for the release of DNA into the cytoplasm, rather than cellular uptake by oligoarginine. Decaarginine-PEG-modified particles had the highest transfection efficiency among a series of oligoarginine compounds, and the superiority of macropinocytosis as a cellular uptake pathway in gene transfection was demonstrated. In intratumoral injection, R4B-polyplex at a lower mixing ratio ± had the highest transfection efficiency among polyplex alone, R10B-polyplex and a commercial gene transfection reagent.

9. Expert opinion

1. *The merits and limitations of oligoarginine-assisted nucleic acid delivery:* Without encapsulation of DNA into particles, decaarginine-modified particles complexed with DNA by just mixing aqueous solutions of oligoarginine lipids and DNA can deliver DNA effectively through different pathway from conventional endocytosis. A limitation of oligoarginine vectors is high cytotoxicity due to high cationic charge, which will be reduced using poor oligoarginine-modified

particles or a specific structure of oligoarginine complexed with DNA.

2. *Analytical method:* A single mechanism has not been established because studies so far lack systematic analysis using different cell types, cargoes, transporter and methods of analysis. A recent study provides direct observation for multiple mechanisms, indicating that the mode by which the octaarginine penetrates the cell membrane appears to be either a multimechanism uptake process or a mechanism different from unimodal passive diffusion or endocytosis [91].
3. *Application for gene delivery:* Non-viral vectors for DNA delivery are at a preclinical stage. However, because DNA has generally similar physicochemical properties, vectors for DNA delivery have high potential for being applicable for treatment of many kinds of disease. Furthermore, modification of oligoarginine such as cysteine-flanked, internally spaced arginine-rich peptides can increase gene expression *in vivo* DNA delivery [92].
4. *For in vivo application:* Oligoarginine for delivery purposes lacks cell specificity. Most CPPs will nonspecifically associate with the membranes of all cell types owing to the fact that most cells express heparan sulfate proteoglycan. The resulting ubiquitous membrane translocation may be beneficial under some circumstances, but may be undesirable in other cases where the targeting of specific cell types or tissues is needed. In the advances of targeted therapy that leaves normal cells undamaged, activatable octaarginine transporters were reported [93]. The therapeutic potential of oligoarginine vectors for systemic DNA delivery is dependent on efficacy of delivery to the target site. For *in vivo* gene delivery, some specially formulated particles, such as with masked oligoarginine or controlled released oligoarginine, and some induction force such as magnet or sonication, may be needed for certain applications. In such cases, the therapeutic potential of these oligoarginine-PEG-lipid particles for gene delivery drives wide gene expression in tumors in the future.

Declaration of interest

The authors state no conflicts of interest and have received no payment in the preparation of this manuscript.

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