Gene Delivery

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A Tailor-Made Specific Anion-Binding Motif in the Side Chain Transforms a Tetrapeptide into an Efficient Vector for Gene Delivery**

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Abstract: *Arginine-rich cell-penetrating peptides are widely utilized as vectors for gene delivery. However, their transfection efficacy still needs to be optimized. To accomplish this, guanidinocarbonylpyrrole groups, which are tailor-made anion binding sites, were introduced into the side chains of tetralysine to obtain the peptide analogue* ***1****. In contrast to the common strategy of adding a lipophilic tail to peptide vectors, this novel method most likely enhances transfection efficacy through more specific interactions between the binding motifs and DNA or the cell membrane. Tetrapeptide analogue* ***1*** *is thus the smallest peptidic transfection vector that has been reported to date. The transfection efficacy of* ***1****, which on average has less than two positive charges under physiological conditions, is even better than that of polyethylenimine (PEI). Furthermore,* ***1*** *exhibits only negligible cytotoxicity, which makes it an interesting candidate for further development.*

The interest in developing new cell-penetrating peptides (CPPs) has grown rapidly in recent years. Hundreds of peptides with various amino acid compositions are capable of translocating through cell membranes.[1] Among them, arginine-rich CPPs have been identified as being highly efficient in terms of cellular uptake[2] and are thus widely tested as gene delivery vectors.[3] However, a minimum of six positive charges is usually required for cellular uptake[2b] and even more positive charges are necessary for efficient gene delivery.[4] Several studies have been published in order to push forward the translocation and transportation ability of arginine-rich CPPs. For example, Futaki[5] et al. demonstrated that N-terminal stearylation of arginine-rich peptides increases the transfection efficacy approximately 100 times, thus making them similarly effective to Lipofectamine, the current gold standard for commercially available nucleic acid transfection agents. Kim[6] et al. reported a cholesteryl-modified arginine nonamer, which could deliver siRNA into cells more efficiently than the original peptide. Recently, we showed that through the addition of a lipophilic tail, peptidic DNA-binding tweezers can be transformed into highly efficient gene delivery vectors.[7] Hence, to date, the main strategy to improve the transportation ability of arginine-rich CPPs has been the addition of a hydrophobic part onto the peptide sequence. However, at least eight to nine arginine residues were still necessary for decent gene transfection even with these amphiphilic oligoarginines. The transfection efficacy and cytotoxicity of these peptides requires further improvement for future applications. Although other cationic peptides such as lysine oligomers[8] have been used for gene transfection, their efficacies are usually even worse than arginine-rich peptides. A new strategy to improve CPPs is therefore much needed. We report herein that through replacement of the guanidinium group in arginine by a tailor-made anion-binding motif, even a tetrapeptide becomes a highly efficient gene transfection vector.

In general, arginine is more efficient than lysine in DNA condensation and membrane transportation because the guanidininum group in arginine can form bidentate hydrogen bonds with anionic groups both in the DNA and in the cell membrane, whereas the ammonium group in lysine only forms unspecific electrostatic interactions. We have previously demonstrated that a weakly basic guanidinocarbonylpyrrole (GCP) moiety can enormously improve the anion-binding efficiency of guanidine in aqueous solution.[9] The binding of anions such as carboxylate or phosphate is enhanced by the formation of a hydrogen-bond-assisted ion pair.[10] We thus hypothesized that the replacement of guanidine with GCP in arginine-rich CPPs should further improve the binding of CPPs to both DNA and the cell membrane, which might result in a better performing vector (Scheme 1).

To test this hypothesis, tetrapeptide analogue **1**, which has four GCP groups instead of guanidine groups, was prepared by Fmoc solid-phase peptide synthesis. The corresponding arginine (**2**) and lysine (**3**) tetramers were also synthesized as controls (Scheme 1). Their binding properties with calf thymus DNA (ctDNA) were first tested by ethidium bromide (EB) displacement assay and isothermal titration calorimetry (ITC). Surprisingly, the differences in DNA binding affinities were negligible. According to the results of the EB displacement assay, the IC_{50} values (concentration of peptide necessary to displace 50% of the initially bound EB) of **1**, **2**, and **3** were almost the same, ranging from 0.85 to 0.89 μM (Table 1). Furthermore, the binding constants for peptide analogue **1** and arginine tetramer **2** with DNA were both in the range of 10^6, as determined by ITC experiments.

However, despite the overall similar binding affinities, the thermodynamic profiles for the interactions of **1** and **2** with DNA were very different. The ITC data (Table 1) show that

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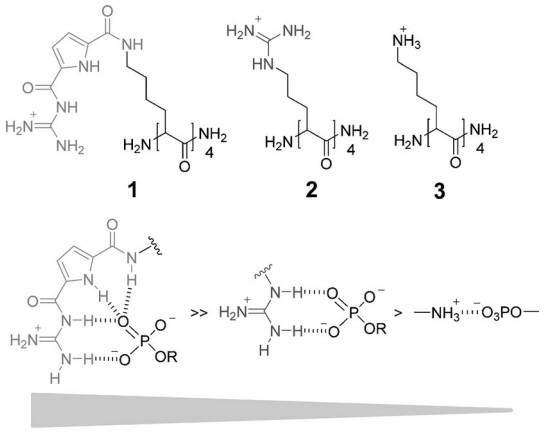

Scheme 1. Oligopeptides **1**–**3**, which were used for the transfection studies. The specificity of the interaction between the cationic groups and oxoanions such as carboxylates or phopshpates decreases in the order **1** ≫ **2** > **3**.

Table 1: Results of ITC and EB displacement assay.

	K_d [L mol^{-1}]	ΔH [kcal mol^{-1}]	$T\Delta S$ [kcal mol^{-1}]	IC_{50}[a] [μM]
1	2.19×10^6	-25.60 ± 0.75	-16.96 ± 0.50	0.85
2	9.23×10^6	-12.34 ± 0.41	-2.84 ± 0.10	0.89
3	n.d.[b]	n.d.	n.d.	0.85

[a] IC_{50}: Concentrations of **1**, **2**, or **3** necessary to displace half of the EB from DNA/EB complex in a fluorescence displacement assay, [DNA] = 3.00 μM, [EB] = 0.75 μM. [b] n.d. = not determined.

the binding of **1** to DNA is characterized by a large enthalpic contribution (−25.60 kcal mol^{-1}), which is partially compensated by a large decrease in entropy (−16.96 kcal mol^{-1}).[11] By comparison, the binding enthalpy of **2** with DNA is less than half of that of **1** (−12.34 kcal mol^{-1}), while the entropy change is negligible (−2.84 kcal mol^{-1}). The large enthalpy–entropy compensation for **1** is a strong indication that **1**, with its tailor-made anion binding site, binds to DNA by forming specific and tight complexes and not only through long-range charge interactions.[12] Based on the ITC data, one can conclude that **1** forms a more specific complex with DNA than the arginine derivative **2**.

The sizes and charges of the aggregates formed by the three peptides **1**, **2**, and **3** with ctDNA were studied by dynamic light scattering (DLS). Their condensation behavior is quite similar (Figure S4 in the Supporting Information). All three peptides form approximately 700–900 nm sized aggregates with DNA (N/P ratios of 40–120), and the aggregates were positively charged under the conditions used for transfection, as confirmed by their corresponding Zeta potentials, which ranged from +9 to +30 mV (Table S1 in the Supporting Information).

Based on these findings, one would not expect that the three peptides would significantly differ in their transfection efficiencies. To test this, the three peptides were directly used to transfect HeLa cells without the addition of any other helper lipids. The widely used commercially available polycationic polymer polyethyleneimine (PEI) was used as a positive control. The cells were examined 24 h after treatment. Despite their similar interactions with DNA in vitro, the transfection efficiency is dramatically different for the three peptides as shown in Figure 1. In agreement with

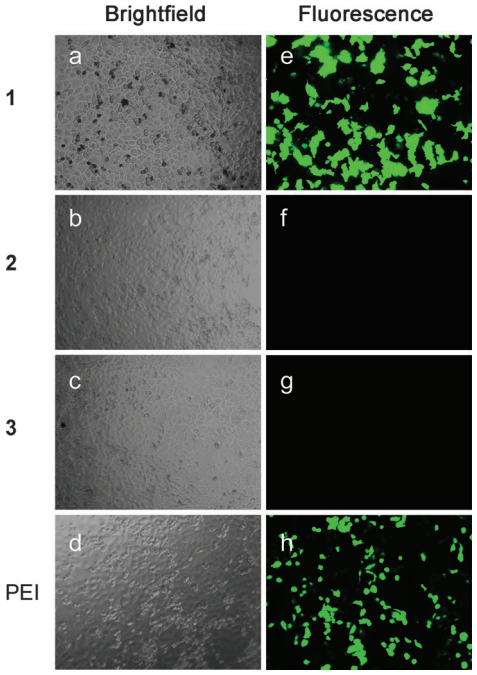

Figure 1. Transfection of 2 μg pF143-GFP plasmid when using **1**, **2**, **3**, or PEI (all 0.15 mM). Brightfield (a–d) and fluorescence (e–h) images of HeLa cells 24 h after transfection with **1** (a, e), **2** (b, f), **3** (c, g), or PEI (d, h)

previous reports, peptides **2** and **3** did not lead to any detectable transfection. In striking contrast to this, however, peptide analogue **1** enabled successful transfection of the cells with a plasmid encoding GFP. The transfection efficacy of **1** was even better than that of PEI, which is one of the current standards in gene transfection (Figure 2B). Considering the low molecular weight and the number of charges of peptide analogue **1** relative to PEI, the transfection ability of **1** is even more remarkable. To the best of our knowledge, this is the first report that such a small peptide with only four amino acids can be used to transfect cells, thus making **1** the smallest peptide based artificial transfection vector reported so far. The results were confirmed in the human embryonic kidney cell line HEK-293 and in murine embryonic fibroblast cell line NIH/3T3 (Figures S6, S7).

Important for any potential applications of a new artificial transfection vector is its cytotoxicity, which for **1** was examined in HeLa cells by measuring metabolic activity as an indicator of cell viability. Toxicity was negligible at 0.15 mM, the concentration at which **1** mediated DNA transfection in our transfection experiments, while in the case of PEI, cell viability was reduced by nearly 70% (Figure 2A).

To elucidate the origin of the dramatically improved transfection efficiency of tetrapeptide analogue **1** compared to the natural tetrapeptides **2** and **3**, transfection experiments

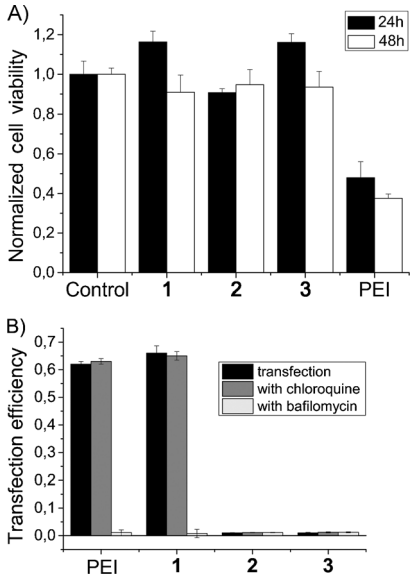

Figure 2. A) Alamar blue cell viability assay. The graph illustrates the fluorescence level of the redox indicator 24 h and 48 h after transfection with **1**, **2**, **3**, and PEI. B) Transfection efficiency for **1**, **2**, **3**, and PEI either with or without the addition of chloroquine (25 μM) or bafilomycin (150 nM).

with the addition of chloroquine or bafilomycin were conducted. The release of the transported DNA from the endosome into the cytosol is often a critical step in gene transfection. The addition of chloroquine, a weak organic base, increases the buffer capacity within the endosome and facilitates cargo release.[13] However, chloroquine had no effect on the cells transfected by arginine tetramer **2** and lysine tetramer **3** (Figure 2B). This suggests that hardly any cellular uptake occurred in the case of **2** and **3**, which is consistent with other reports that short cationic peptides with less than six charges are not able to translocate through cell membranes.[2a] Thus, the main reason for the increased transfection efficiency of peptide analogue **1** relative to **2** and **3** is its enhanced cellular uptake, which most likely originates from a more specific binding interaction between the GCP group in **1** and negatively charged groups on the cell membrane.

In line with this finding, Rothbard et al. reported that decreasing the hydrogen-bonding ability of an arginine octamer dramatically reduces its cellular uptake. An arginine octamer with two additional methyl groups on each guanidinium group completely lost its cellular uptake properties although the number of positive charges was identical to that of the unmodified peptide.[14] Hence, specific binding interactions with the cell membrane seem to play an important role in the cellular uptake mechanism of CPPs. It has also been reported that the interaction of arginine with membrane-associated proteoglycans is crucial to the cellular uptake of arginine-rich CPPs.[15] The structures of arginine oligomers and their binding to cell-surface proteoglycans could determine their cellular uptake.[16] This also leads to significantly different outcomes in cellular internalization and gene transfection.[8]

Besides the better uptake of **1**, the low pK_a value of the GCP group also results in an increased buffering capacity within the endosomes, which facilitates endosomal escape by the proton-sponge effect.[7] Accordingly, DNA transfection by **1** was completely inhibited by bafilomycin, which blocks the endosomal acidification process (Figure 2B). In consequence, the DNA stays trapped within the endosome and is degraded there before transfection occurs. The replacement of arginine by an artificial GCP-containing amino acid thus not only enormously enhances the endosomal uptake of peptide/DNA complexes by cells, but also facilitates the necessary release of the DNA from the endosomes to ultimately enable gene transfection.

In summary, this research highlights the potential of specific tailor-made anion binding sites, such as our GCP group, to improve CPP-mediated gene delivery. Such supramolecular binding motifs enable more specific interactions not only with the DNA but also with the cell membrane. This enormously enhances the cellular uptake of the corresponding peptide/DNA polyplexes relative to peptides with only natural amino acids. Tetrapeptide **1**, which contains only four of these artificial GCP groups, is a highly efficient transfection vector. It is not only more efficient than the commercial reagent PEI but also shows significantly lower cytotoxicity. Further studies of the role of specific binding interactions in gene delivery by cell-penetrating peptide are in progress.

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