Focused Libraries

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Utilizing Combinatorial Chemistry and Rational Design: Peptidic Tweezers with Nanomolar Affinity to DNA Can Be Transformed into Efficient Vectors for Gene Delivery by Addition of a Lipophilic Tail**

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Efficient delivery of genetic information into cells is crucial for many biotechnological and medical applications like genome analysis by means of RNAi[1] and gene therapy.[2] Recombinant viruses, though very effective, have several severe drawbacks (toxicity, immunogenicity, limited scale-up) at least for medical applications.[3] The use of nonviral vectors can in principle circumvent these problems,[4] and two main classes are currently being explored: 1) oligo- and polycationic carrier systems, like cell-penetrating peptides (CPP)[5] and the polymer polyethylene imine (PEI),[6] and 2) cationic amphiphilic lipids, which have become the most promising chemical vectors for cell transfection in biotechnology.[7] The most prominent example is Lipofectamine 2000,[8] a mixture of the synthetic polycationic lipid DOSPA and the neutral helper lipid DOPE.[9] These compounds self-assemble into positively charged liposomes that bind to the negatively charged DNA based on unspecific charge interactions. The resulting polycationic particles (lipoplexes) interact with the negatively charged cell surface and are taken up into the cell mainly by endocytosis.[10] The lipid parts of the lipoplexes then destabilize the endosomal membrane, thus releasing the DNA into the cytoplasm, from where it is transported into the nucleus.[11] As artificial transfection vectors still have serious limitations[12] that prevent their application in gene therapy, the development of more efficient agents is needed.[13] Several studies also including the screening of combinatorial libraries have focused on the importance of both the lipophilic part[13c] and the cationic head group. Nonetheless Lipofectamine, discovered in 1993, remains state of the art. Most artificial transfection vectors studied so far employ unspecific polyamine-derived (i.e. polycationic) head groups. We now report a new type of artificial transfection vector obtained from the combination of specific DNA-binding molecular tweezers (as a cationic head group) with a lipophilic tail.

Recently we observed that a cationic tweezer[14] composed of two dipeptide arms (Figure 1: **1a**: R = H; AA^1 = Phe; AA^2 = Lys; AA^3 = none) and the weakly basic guanidinocarbonyl pyrrole moiety (GCP)[15] binds to generic DNA but only with micromolar affinity. It also transferred DNA into cells but did not lead to transfection.[16] The multiarm design (two or more arms) was shown to be necessary for effective DNA binding, as one-armed analogues of **1a** bound less effectively to DNA by two orders of magnitude and also did not lead to transfection.[17] Starting from this initial observation, we will demonstrate herein that:

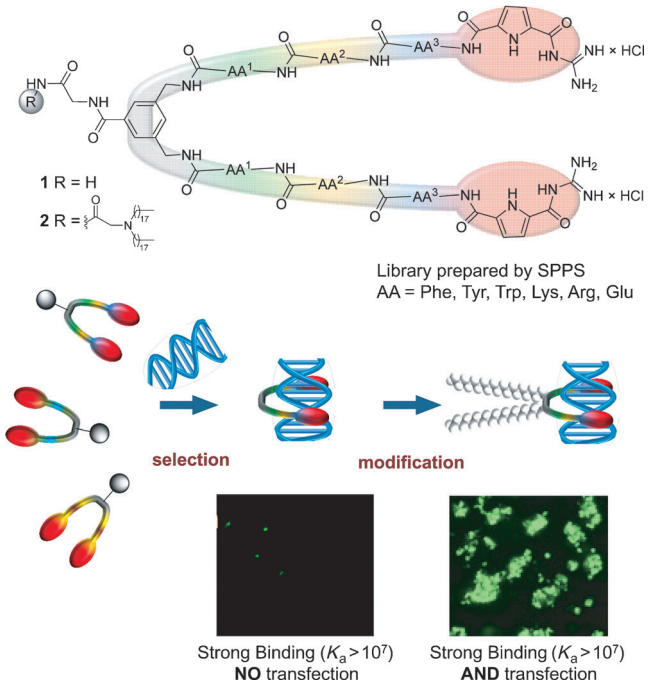

Figure 1. Screening a combinatorial library of 259 molecular tweezers **1** composed of two identical peptide arms (amino acids AA^1–AA^3) and a guanidinium-based oxoanion binding motif (red) identified DNA-binding ligands that had with nanomolar affinity but only little transfection efficiency. The addition of a lipophilic tail transformed these highly affine ligands into efficient and nontoxic transfection vectors.

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1) By screening a small but focused combinatorial library of 259 molecular tweezers of type **1** (varying amino acid sequence and arm length) we were able to increase the affinity of the ligands by more than three orders of magnitude compared to our original DNA binder **1a**. Interestingly, the best binders with now nanomolar affinity did not have the highest possible charges.
2) With increasing affinity of the ligands, their ability to transfect cells also increases, although transfection levels remained low even for the best DNA binders with nanomolar affinity.
3) By combining the optimized DNA ligands with a lipophilic tail a new class of artificial transfection vectors was obtained, which is similarly efficient as the current gold standard Lipofectamine 2000. But in contrast to Lipofectamine our vectors do not require any additional helper lipid.

To improve the affinity of molecular tweezers **1** for DNA binding, a combinatorial library of 259 related tweezers was synthesized on solid support using a split-mix protocol. The symmetrical divalent design of the original ligand and the GCP head groups were retained as we know from our previous work that related linear tri- and tetrapeptides bind only very weakly to DNA and do not lead to transfection.[17] As building blocks for the arms we chose three types of amino acids: 1) the aromatic amino acids phenylalanine, tyrosine, and tryptophan, which are known to be able to "dig into" the cell membrane and thus might facilitate membrane penetration and improve binding by additional intercalating interactions with the DNA;[18] 2) the cationic moieties lysine and arginine, which not only bind to the negatively charged phosphate backbone of nucleic acids, but are also able to interact with negatively charged groups on the cell surface such as heparin sulfate;[19] 3) glutamic acid, which served as a negative control in the construction of the combinatorial library. The length of the peptidic arms was varied from zero to three amino acids. This library design also guarantees that the original DNA ligand **1a** with the Phe-Lys dipeptide is also part of the library, thus allowing for a direct comparison of the binding affinities.[16] Hence, the library comprises in total 259 members ($6^3 + 6^2 + 6 + 1$). In earlier work on oligopeptide recognition by artificial chemical receptors we could already show that such a small but focused library can be as effective with respect to the screening results as a much larger but random one.[20] Another advantage of small libraries is that they can be screened in a way that allows for quantitative assessment of all library members and not just the qualitative identification of a few active hit structures by means of yes/no anwers.[21]

After completion of the library by solid-phase peptide synthesis (SPPS) (for details see the Supporting Information), all library members were screened for their DNA-binding affinity towards the plasmid pF143-GFP (6238 base pairs (bp)). This plasmid encodes for a green fluorescence protein and was also used for the cell transfection experiments.[22] An ethidium bromide (EB) displacement assay with all 259 solid-phase-bound library members was performed using a high-throughput fluorescence microtiter plate reader (see the Supporting Information).[23] In this screening assay, a 4:1 mixture of DNA and EB was mixed with aliquots of the individual solid-phase-bound library members and the resulting fluorescence intensity was measured as an indicator for their binding affinity.

Within this screening our original ligand **1a** showed only modest affinity. Roughly half of the library members bind significantly better to the DNA than **1a**. The ten most affine library members are listed in Table 1. Within the top ten, arginine is the predominant amino acid (13 occurrences). Lysine is found six times and aromatic amino acids in total nine times. As expected, glutamic acid is not present at all in the best binders. In general, the ligands of highest affinity feature high positive charges (+5), but interestingly not the highest possible charge (+7). As one would expect for a polyanionic substrate, high positive charges seem to be advantageous for DNA binding, but obviously they are not the only criterion.[24] There is even a ligand with just three positive charges and only two amino acids present in the top ten (Arg-Tyr-GCP). This strongly suggests that not only unspecific charge interactions between the nucleic acid and the ligand occur but rather specific binding and complexation takes place. Tyr and Trp are both able to intercalate into DNA which could be one reason why tweezers containing these amino acids outperform their higher charged congeners that do not contain aromatic amino acids.[18c–f] Other possible explanations could be shielding effects by the aromatic side chains and additional van der Waals contacts, for example, within the groove, which are more favorable for the binding event than additional charges.

Table 1: The ten best pF143-GFP binders identified from the on-bead EB displacement screening assay.

AA^1	AA^2	AA^3	Charge[a]	Rel. fluorescence [%]
Trp	Arg	Lys	+5	9
Trp	Lys	Arg	+5	13
Lys	Phe	Arg	+5	15
Lys	Lys	Phe	+5	16
Arg	Tyr	–	+3	18
Arg	Lys	Tyr	+5	18
Arg	Tyr	Arg	+5	19
Arg	Trp	Arg	+5	19
Arg	Phe	Arg	+5	19
Arg	Arg	–	+5	19

[a] Charges are mean values calculated for pH 7 with the known pK_a values for Arg (12.5), Lys (10.5), and GCP (7.0).

In order to verify the results of the on-bead screening, four ligands were resynthesized on a larger scale for binding studies in solution. The synthesis was carried out following a microwave-assisted SPPS procedure.[21] The selected ligands comprise the two best performing sequences of the on-bead screening, Trp-Arg-Lys-GCP (**1b**) and Trp-Lys-Arg-GCP (**1c**), as well as their sequencial isomer Lys-Trp-Arg-GCP (**1d**), and as a negative control Glu-Trp-Arg-GCP (**1e**). The original sequence Phe-Lys-GCP (**1a**) from our previous study was also resynthesized for comparison. The DNA-binding affinity of these tweezers in solution was determined using an

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EB displacement assay as well as isothermal titration calorimetry (ITC). Representative experiments are shown in Figure 2 (for all others, see Figures S17–S19 in the Supporting Information).

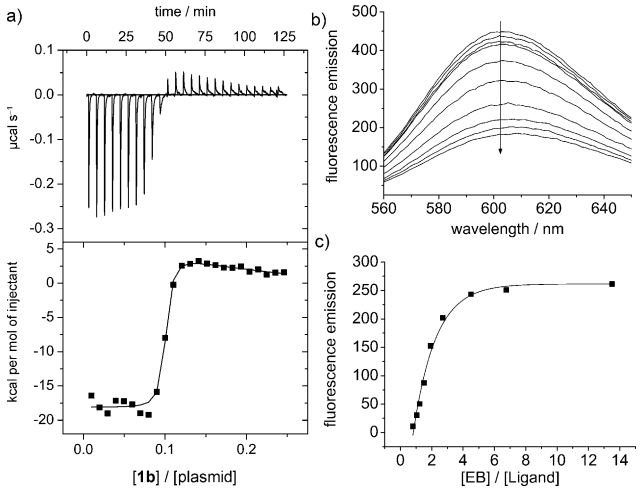

Figure 2. Solution-phase DNA-binding studies with tweezer **1b** in sodium cacodylate buffer (0.01 M) at pH 7. a) ITC experiment: **1b** (0.1 mM) was titrated into a solution of pF143-GFP (0.035 mM); the titration was corrected for dilution of the ligand and fitted with a two-site model. b) EB fluorescence displacement titration. c) Section at 600 nm plotted against the ratio of EB/**1b** with the corresponding fit (bottom).

Table 2: Comparison of binding studies in solution and on-bead with pF143-GFP plasmid DNA in neutral aqueous solution (cacodylate buffer, 0.01 M) and transfection efficiency 48 h after treatment of HEK293T cells with plasmid DNA and **1a**–**1e**. The sequences are reported from AA^1 to AA^3 with Arg = R, Lys = K, Phe = F, Trp = W, and Glu = E.

Compound	**1a**[14]	**1b**	**1c**	**1d**	**1e**
Sequence	FK	WRK	WKR	KWR	EWR
IC_{50} [equiv][a]	1.85	0.51	0.55	0.36	31.2
log K_{a2}[b]	6.30	8.74	8.42	9.22	5.82
Transfection Efficiency [%]	<1	6.0	6.5	9.6	<1

[a] IC_{50}: the amount of ligand in equivalents that is necessary to displace half of the EB from a DNA/EB complex in a fluorescence displacement assay; [DNA] = 3.00 µM (DNA concentration refers to base pairs), [EB] = 0.75 µM. [b] Determined by ITC titrations of ligand (**1b**, **1d**, **1a**: 0.1 mM; **1c**: 0.125 mM; **1e**: 0.2 mM) to DNA solutions (**1b**: 0.035 mM; **1c**: 0.05 mM; **1e**: 0.03 mM; **1d**, **1a**: 0.04 mM). Deviations in IC_{50} values: **1a**: ±0.12, **1b**: ±0.03; **1c**: ±0.04; **1d**: ±0.04; **1e**: ±0.7. Deviations in transfection efficiencies: **1b**: ±2.1, **1c**: ±2.3, **1d**: ±2.8.

The results obtained with the two methods (Table 2) are in excellent agreement. The new ligands **1b**–**1d** feature binding constants for the plasmid that are two to three orders of magnitude higher than that of the original ligand **1a**. Hence, with this combinatorial approach the binding affinity of such peptide-derived tweezers towards DNA was improved tremendously.

The ITC data show that the binding of the tweezers to the plasmid DNA can be described best as a two-step process (see the Supporting Information). The first binding event is endothermic and entropy driven. The binding constants are rather low ($K_a \approx 10^5$–$10^6\,M^{-1}$) and quite similar for all the different ligands. This is a characteristic signature for unspecific backbone binding based on long-range charge interactions and the displacement of multiple associated monocations from the DNA upon binding of a single polycationic ligand.[25] The second binding event is exothermic with a small unfavorable entropy contribution. Such a signature is characteristic for groove-binding proteins.[26] For this second binding event the affinity of the different ligands differs by roughly three orders of magnitude. Hence, in contrast to most cationic head groups used so far in artificial transfection vectors, which bind to DNA mainly based on unspecific charge interactions, our tweezers form specific complexes with DNA. This not only allows for higher affinity but in principle could also lead to sequence-specific DNA binding in the future.

Screening a focused combinatorial library therefore made it possible to enhance the DNA binding affinity of this type of molecular tweezers from micromolar (for the first prototype **1a**) to nanomolar affinity. But even though the binding affinity was increased by three orders of magnitude the transfection efficiency of the new ligands remained rather low (Table 2). Transfection experiments were performed by incubating HEK293T cells with a mixture of pF143-GFP plasmid DNA (2 µg) and the DNA ligands **1b**–**1e** (0.24 mM). Successful transfection leads to the expression of a GFP protein and thereby to green fluorescing cells which were monitored 48 h after transfection. The highest transfection efficiency was achieved by **1d** with 9.6%, followed by **1c** (6.5%) and **1b** (6.0%). Neither the original ligand **1a** nor the negative control **1e** were able to transfect cells (<1%).[13]

Colocalization studies with HeLa cells showed that not DNA uptake but rather the release and further processing of the internalized genetic information is responsible for the low transfection efficiencies. Membranes were first marked with the lysosome-associated membrane protein 1 (Lamp1) tagged with a red fluorescence protein. Afterwards the same cells were transfected with green fluorescently labeled DNA with the help of the gene carriers **1a**–**1e**, and analyzed 2 h later by live-cell confocal microscopy (Figure S22 in the Supporting Information). In contrast to the negative control **1e** all other tweezers **1b**–**1d** were able to shuttle the labeled DNA into the cell. However, despite the relatively high amount of DNA within the cell the resulting transfection efficiency is still rather low (ca. <10% with this cell type). These findings suggest that the molecular tweezers are able to shuttle nucleic acids into the cells but that the DNA then remains trapped within the endosome where it is degraded. If this truly is the case, one should be able to vastly improve the transfection capabilities of these molecules by covalent attachment of lipophilic alkyl chains, which will convert these molecules into cationic lipid amphiphiles. The lipophilic tails should be able to facilitate endosomal release after cell uptake and thus improve transfection efficiency.[11,27] Similarly, most artificial transfection vectors (including the gold standard Lipofectamine) require the addition of the neutral helper lipid DOPE for efficient transfection. To test this hypothesis amphiphilic analogues **2** with two C_{18} alkyl chains attached to the

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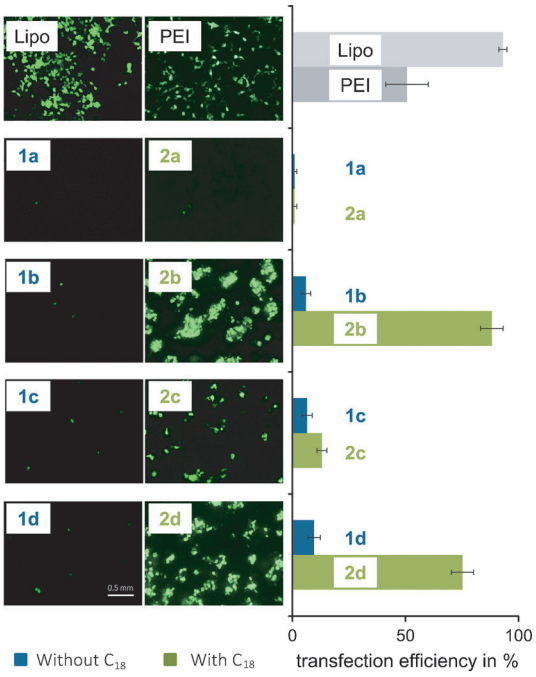

Figure 3. Comparison of transfection efficiencies with and without C_{18} derivatization; fluorescence images show HEK293T cells 48 h after transfection with 2 μg pF143-GFP plasmid and **1a**–**1d** (0.24 mM), **2a**–**2d** (0.09 mM), PEI (0.24 mM), and Lipofectamine 2000 (0.5 μL + 0.2 μg plasmid). Scale bar: 0.5 mm.

molecular tweezers (R = 2-(dioctadecylamino)-2-oxoethyl, Figure 1) were synthesized. As illustrated in Figure 3, these amphiphilic C_{18}-derivatized transfecting agents, namely **2b** and **2d**, clearly outmatch their parent compounds (**1b**, **1d**) even at significantly lower concentrations (0.09 instead of 0.24 mM, respectively). With no need for the addition of the helper lipid DOPE or any special requirements for liposome preparation as otherwise often observed for lipid-based artificial gene vectors,[24] the best amphiphilic vector **2b** (88.2%) even outmatches PEI (51.0%) and is as efficient as Lipofectamine 2000 within standard deviation (93.1%). Hence, the amphiphilic character of the novel DNA ligands dramatically improves their properties as transfecting agents, most probably by enhancing their capability for endosomal escape. Also other human and murine cell lines (HeLa, MCF-7, NIH/3T3; see the Supporting Information) can be efficiently transfected with our gene carriers.

Finally, we also assessed the cytotoxicity of **2a**–**2d** by measuring the metabolic activity of HEK293T cells after transfection with the help of an Alamar Blue assay (see the Supporting Information). Pleasingly, our new amphiphilic DNA carriers **2b** and **2d** proved to be not significantly cytotoxic, making them a valuable addition to the field of artificial transfection vectors.

In conclusion we have developed a new method for the identification of highly efficient nonviral, lipid-based vectors for gene delivery. We optimized a short sequence of amino acids (1–3 AAs) within a tweezers-like molecule tipped with a tailor-made anion binding group for affinity towards plasmid DNA by combinatorial means, thereby achieving nanomolar binding constants ($K_a > 10^9\,M^{-1}$). Interestingly, specific affinity was not determined by overall charge alone, implying that specific complexation of the DNA by the tweezers occurs. Even though these high-affinity ligands are able to shuttle DNA into cells, gene transfection efficiency remained low. To facilitate endosomal escape, the optimized sequences were used as head groups for cationic lipids and two lipophilic C_{18} hydrocarbon chains were covalently attached to the peptide tweezers. These novel lipid-based cationic vectors such as **2b** and **2d** proved to be very efficient in transfecting various cell types and were as good or even better than currently used transfection agents such as Lipofecatmine and PEI. Importantly, our new high-affinity amphiphilic DNA binders function as single-compound transfection agents as they do not require the addition of any helper lipid. They are non-cytotoxic and accessible with only moderate synthetic effort, making them overall a highly interesting and easy-to-use new class of transfection agents that might even have the potential for further development into sequence-specific "DNA shuttles".

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