Gene Transfection

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Incorporation of a Non-Natural Arginine Analogue into a Cyclic Peptide Leads to Formation of Positively Charged Nanofibers Capable of Gene Transfection

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Abstract: *Functionalization of the tetracationic cyclic peptide $(Ka)_4$ with a single guanidiniocarbonyl pyrrole (GCP) moiety, a weakly basic but highly efficient arginine analogue, completely alters the self-assembly properties of the peptide. In contrast to the nonfunctionalized peptide* ***2****, which does not self-assemble, GCP-containing peptide* ***1*** *forms cationic nanofibers of micrometer length. These aggregates are efficient gene transfection vectors. DNA binds to their cationic surface and is efficiently delivered into cells.*

Peptide-based gene transfection vectors have attracted increasing interest as a result of their ease of synthesis, lower toxicity (at least compared to polymeric transfection vectors), and versatility of functionalization.[1] However, peptidic vectors often possess poor endosomal release abilities which hinder any further applications since most cargo DNA stays entrapped in the endosome until it is degraded by enzymes. As a consequence, most peptide-based gene transfection vectors suffer from low transfection efficiency compared to other types of artificial vectors such as lipofectamine.[2] Although many strategies have been developed to improve their efficacies (most often by making them amphiphilic, for example, by adding a lipophilic tail), transfection abilities are still far from satisfying.[3] As well as individual peptides, self-assembled peptide aggregates have also been tested as gene transfection vectors. For example, M. Yolamanova et al. recently reported that an amphiphilic 12 amino acid peptide derived from the HIV-1 glycoprotein gp120 forms cationic nanofibers which boost virus-mediated gene transfection.[4] Only the nanofibers, not individual peptides, enhanced gene transfection, probably by functioning as an "electrostatic nanobridge" between the virus and the cell membrane. It has also been reported that nanotubes formed from cationic dipeptides bind single-strand DNA. Upon dilution these nanotubes rearrange into vesicles which are taken up by cells.[5]

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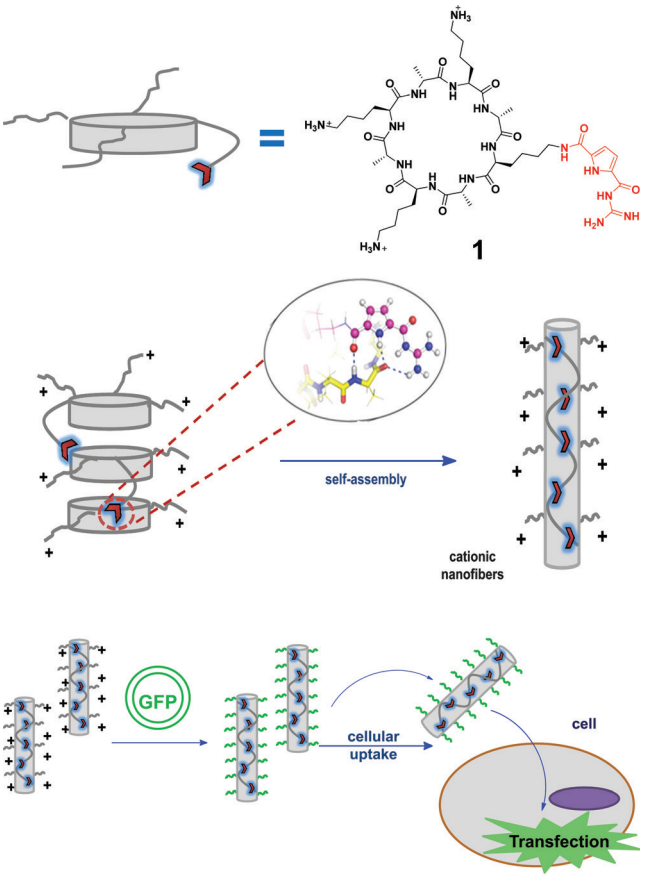
Supramolecular chemistry has developed several strategies to achieve efficient and also controlled peptide assembly.[6] Amphiphilic peptides can readily form peptide fibers in aqueous solutions.[6d,7] Additionally, naturally occurring peptides, such as amyloid and coiled-coil peptides, are interesting building blocks in constructing peptide-based nanotubes.[8] In particular, cyclic peptide nanotubes have become a highly interesting scaffold for a variety of applications since their first discovery in the nineties.[9] The rather robust self-assembling properties of cyclic peptides constructed from alternating D and L amino acids make them interesting candidates for application, for example, as transmembrane ion channels, stimuli-responsive nanomaterials, and antibacterial agents.[10] However, because of the inherent self-assembling mechanism, cyclic peptide nanotubes normally cannot be positively charged under physiological conditions since charge repulsion then prevents tube formation. Unfortunately, positive charges are usually critical for DNA binding and also gene transfection as a result of the anionic nature of DNA. Although several reports have demonstrated excellent control of the interior properties of cyclic peptide nanotubes, modification of their exterior charge states has not yet been realized especially in aqueous conditions.[11] Thus, cyclic peptide nanotubes have not been used for DNA binding or even gene transfection to date.

We have introduced the weakly basic guanidiniocarbonyl-pyrrole moiety (abbreviated GCP) as a strong anion-binding site.[12] Recently, we showed that functionalization of tetralysine with this tailor-made anion-binding site transformed even this small tetrapeptide into a highly efficient gene delivery vector.[13] Thus, we hypothesized that functionalization of a cationic cyclic peptide with our GCP group could significantly alter its self-assembly and DNA-binding features.

To test this hypothesis, cyclic peptide **1** was designed. It consists of four L-lysine (K) residues and four D-alanine (a) residues (Scheme 1). One of the lysine side-chains was functionalized with our GCP group. The synthesis was achieved by Fmoc solid-phase peptide synthesis (see the Supporting Information for details; Fmoc = 9-fluorenylmethoxycarbonyl). Peptide **2** without the GCP modification was synthesized as a control peptide. The self-assembling abilities of both peptides were first tested by AFM and TEM. As expected, peptide **2** does not form any ordered nanostructure at pH 7.4 in water (Figure S3). It is most likely that charge repulsion between the positively charged ammonium groups in the lysine side-chains hindered the hydrogen-bond-



Scheme 1. Peptide **1** self-assembles into cationic nanorods which are able to shuttle DNA into cells.

induced aggregation of the cyclic peptides, which is consistent with previous reports.[14] The result was also confirmed with dynamic light scattering (DLS) in solution as no aggregates could be detected.

However, to our surprise, modification of peptide **2** with only a single GCP moiety completely changed its self-assembly behavior. DLS clearly showed that peptide **1** formed large aggregates in buffered water (cacodylate buffer) at pH 7.4 (see Figure S2 in the Supporting Information), a pH value at which the weakly basic GCP might not yet be fully protonated but the remaining three lysine residues are (amine: $pK_a \approx 10$; GCP: $pK_a \approx 7$). Therefore, as expected at this pH value the aggregates are overall positively charged as determined by zeta potential measurements (+4.08 mV). However, in both AFM and TEM images, tubular structures as typically seen for other self-aggregated cyclic peptides were observed (Figure 1A; Figure S4). The lengths of these nanorods were in the range of micrometers with widths of approximately 100 nm. It can also be deduced that the observed aggregates consisted of several smaller nanorods with widths around 7 nm. The exact internal structure of these aggregates remains so far, however, unclear. Nevertheless, as far as we know, this is the first report that a positively charged cyclic peptide forms this type of nanofiber.

Molecular modelling suggested that the GCP group could stabilize a stack of peptides by binding to the backbone of an adjacent cyclic peptide (Figure 1B). Thus, the GCP groups

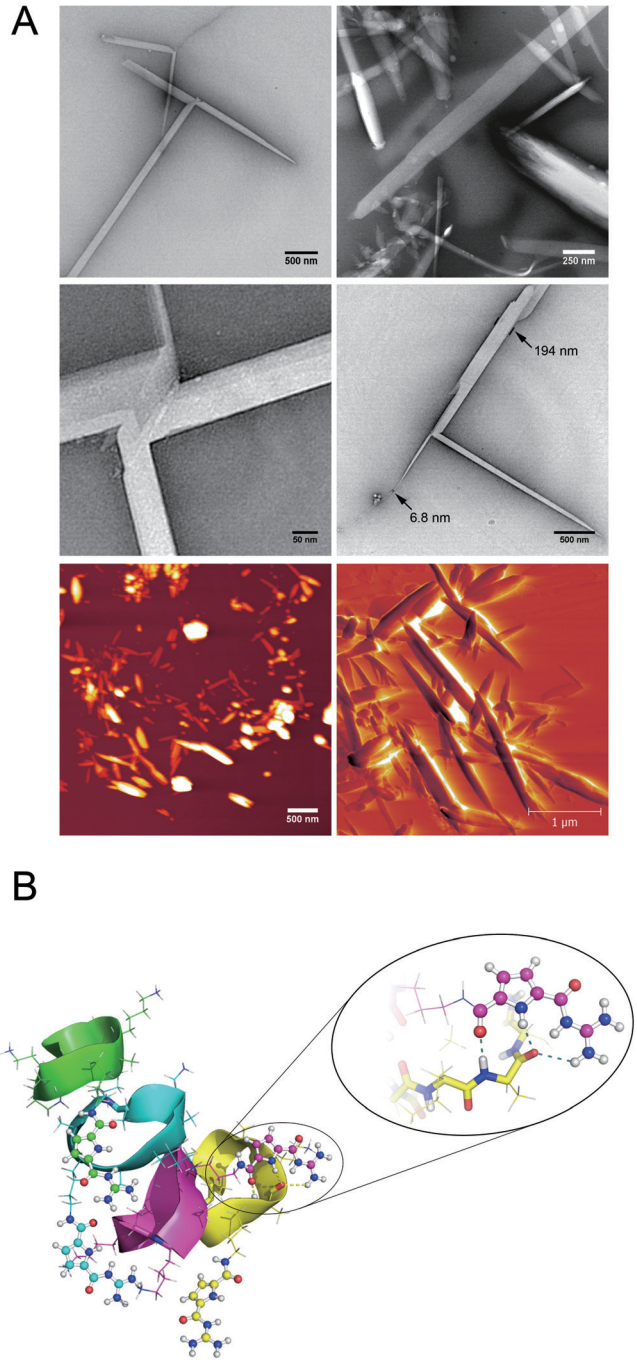


Figure 1. A) TEM (upper and center panels) and AFM (lower panels) images of peptide **1** (0.4 mM, pH 7.4) in water. B) Molecular modelling of peptide **1**. Scale bars in (A): Upper left, middle right, and lower left = 500 nm, upper right = 250 nm, middle left = 50 nm, lower right = 1 µm.

might function as "extra clamps" between the stacked cyclic peptides (see also Scheme 1). This binding interaction could provide enough extra stabilization to overcome the charge repulsion of the remaining lysines allowing nanotube formation even for a positively charged cyclic peptide. Notably, peptide **1** is also significantly more hydrophobic than peptide **2** as evidenced, for example, through the corresponding HPLC elution profiles (Figure S12). This property may also

favor the self-aggregation of peptide **1** in water relative to peptide **2**. However, the exact mechanism of self-assembly and the role of the GCP group still need to be investigated in more detail.

As the aggregates are cationic overall, we next tested their interaction with calf thymus DNA (ctDNA) as model DNA. The addition of the DNA to the peptide nanofibers did not change their size or their tubular structure as can be seen from the DLS measurements (Figure S2) and TEM images (Figure 2, upper panels). More importantly, as the TEM

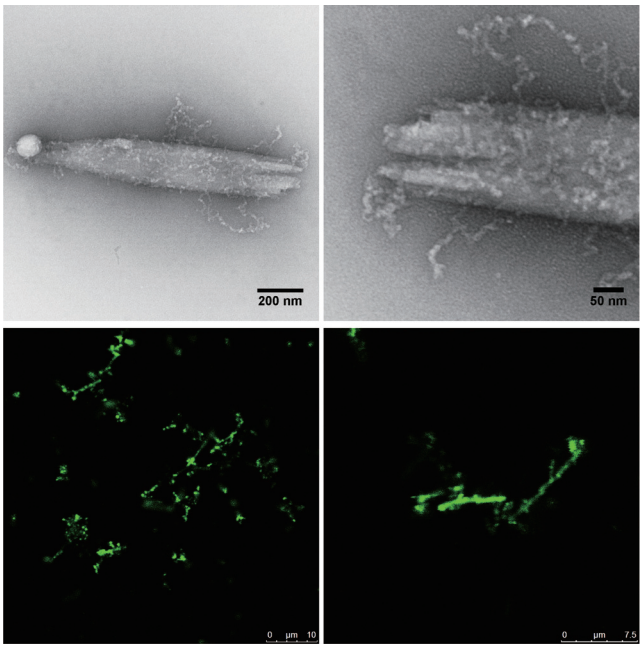


Figure 2. TEM (negative stain) images (upper panels) of peptide **1**/ctDNA mixture. Peptide **1** (0.4 mM) was mixed with ctDNA (0.02 mM) in water at pH 7.4. The lower panels show confocal microscopy images of the peptide **1**/labeled-plasmid-DNA mixture. Scale bars: Upper left = 200 nm, upper right = 50 nm, lower left = 10 µm, lower right = 7.5 µm.

images show the negatively charged DNA attaches to the surface of the cationic nanorods. To further confirm the results, we labeled plasmid DNA with a green fluorophore and mixed it with peptide **1**. The resulting mixture was directly examined with confocal fluorescence microscopy. Figure 2 (lower panels) clearly showed that labeled DNA was successfully attached onto the peptide aggregates (see also Figure S8).

As DNA clearly attaches to the nanofibers, we determined whether they could be applied as vectors in gene delivery. Therefore, green fluorescent protein (GFP) plasmid, a typical reporter gene in transfection experiments, was mixed with the peptide aggregates. The resulting mixture was directly used to transfect Hela cells. No other helper molecules such as lipids were added, which are often required for other artificial transfection vectors to enable gene transfection. The widely used, commercial available polycationic polymer polyethyleneimine (PEI) was used as a positive control.

Transfection results were imaged 24 hours after incubation with Hela cells. As shown in Figure 3 A, peptide **2**, being even more positively charged than **1** but not forming any nanotubes, was not able to transfer plasmid into cells. In striking contrast to this, the aggregates formed by peptide **1** successfully transported the GFP plasmid into cells as seen by the expression of the green fluorescent protein. Moreover, the transfection efficacy of peptide **1** was comparable to PEI (Figure 3 B). Furthermore, peptide **1** is even less cytotoxic than PEI (Figure S7). To our knowledge, this is the first report that a cyclic peptide self-assembles into nanofibers which can be applied as gene transfection vectors. The transfection results were also confirmed in human embryonic kidney cell line HEK-293 (Figure S4).

To further elucidate the mechanism of gene transfection by peptide **1**, transfection experiments with the addition of

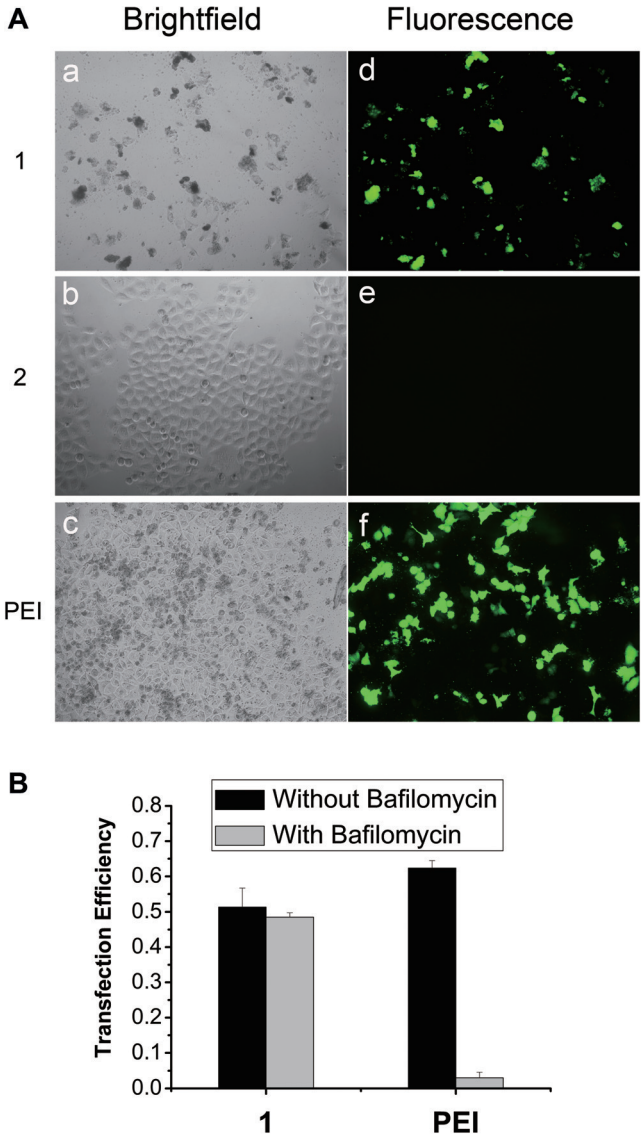


Figure 3. A) Transfection results with 2 µg pF143-GFP plasmid using **1**, **2**, or PEI (all 0.4 mM). Brightfield (a–c) and fluorescence images (d–f) of Hela cells 24 h after transfection with **1** (a, d), **2** (b, e), or PEI (c, f). B) Co-transfection of **1** and PEI with or without bafilomycin treatment (150 nM).

bafilomycin were conducted. In case of an endosomal uptake bafilomycin can entrap the cargo within the endosomes by inhibiting the acidification of the endosomes, thereby preventing transfection. It has been demonstrated that endocytosis is the major uptake pathway for PEI-mediated gene transfection. Thus, when the cells were treated with bafilomycin prior to transfection, the endocytosis pathway was blocked and accordingly transfection with PEI was completely inhibited (Figure 3B). However, bafilomycin did not have any effect on the gene transfection by peptide **1**. This suggests that it is unlikely that an endocytotic pathway is responsible for the cellular uptake process in gene transfection mediated by **1**. Thus, a more reasonable explanation is that nanotubes formed by cyclic peptide **1** function as a bridge between DNA and the cell membrane so that DNA molecules could enter into cells without the involvement of endocytosis.[4] A more detailed study on the transfection mechanism will follow in due course.

In summary, the functionalization of the cyclic peptide $(Ka)_4$ with a single GCP group resulted in the formation of peptide aggregates, most likely nanotubes. Interactions between the GCP group and the cyclic peptide backbone might help stabilize the tubular structure by offsetting charge repulsions. Thus, a positively charged cyclic peptide nanofiber was obtained under physiological conditions. The resulting aggregates showed an astonishing ability in gene transfection through a non-endocytic cellular uptake pathway. Further studies of the role of GCP modifications are in process.

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