Review

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A new family of peptide–nucleic acid nanostructures with potent transfection activities‡

Burkhard Bechinger,[a]* Verica Vidovic,[a] Philippe Bertani[a] and Antoine Kichler[b,c]

A family of His-rich peptides has been shown to complex DNA and efficiently deliver these nucleic acids into eukaryotic cells. Therefore, these nanoscale complexes have potential applications in gene therapy. Here, we review a number of spectroscopic and biophysical investigations aimed at characterizing the supramolecular interactions of the peptides with the nucleic acids and when overcoming the membrane barriers of the cell. Furthermore, solid-state NMR distance measurements for the first time reveal close interatomic distances between the amino acid side chains and the DNA phosphates within the transfection complex. A recent study indicates that the peptides are also potent transfectants of siRNAs and they could thereby be of potential interest for gene silencing therapies using these compounds. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-state NMR; isothermal titration calorimetry; siRNA; DNA; histidine-rich peptide; antimicrobial peptide; membrane interaction; cell-penetrating peptide

Introduction

The capacity to deliver nucleic acids into cells has profoundly changed fundamental and applied research. Indeed, besides allowing one to investigate the effect of expression of a given protein in a cell, it has also allowed the concept of gene therapy to emerge. The objective of the latter is to deliver a therapeutic gene into cells for the treatment of acquired and genetic diseases. However, as DNA is a negatively charged macromolecule that does not enter cells, the success of gene therapy will depend on our capacity to develop efficient and safe DNA vehicles.

One delivery approach consists in exploiting the properties of viruses such as adeno-associated virus (AAV), which corresponds to a protein/DNA supramolecular assembly about 25 nm in size. This is currently the most widely used system, including in clinical trials. However, these viral vectors are not devoid of limitations: for example, the maximal size of DNA fragment they can introduce into a cell is <5 kb. All other approaches are collectively summarized under the term 'nonviral gene delivery systems' and use designed molecules. The approach that has attracted the greatest attention consists in using synthetic cationic compounds [1–3]. The rationale of this strategy was simple, namely, mimicking the properties of histones, i.e. condensing the DNA in order to obtain nanometric particles. Indeed, most cationic compounds are able to compact DNA. However, DNA condensation is not sufficient for efficient transfection of eukaryotic cells. In fact, the (ideal) transport system should, in addition, be able to protect DNA against enzymatic degradation, allow for the cellular uptake of the complexes, facilitate the endosomal escape of the plasmid and lastly, favor the nuclear delivery.

In the last 20 years, a great variety of molecules have been synthesized, ranging from polypeptides such as polylysine to synthetic lipids, polymers, dendrimers, nanoparticles and peptides. Notably, among all, the latter family is the least explored, although peptides allow product identification and quality control as well as the possibility of a reproducible and scalable production process, which are all important properties for future biomedical applications.

Cationic Peptides for Gene Delivery

In the 1990s, one of the most popular transfection agent was polylysine, although this homopolymer performed poorly when mixed only with DNA [4]. But pioneering work by Ernst Wagner's group showed that the transfection efficiency can be greatly enhanced by adding fusogenic anionic peptides to the polylysine–DNA complexes. In particular, these authors used peptides derived from the *N*-terminal segment of the HA-2 subunit

* *Correspondence to: Burkhard Bechinger, Faculté de chimie, Institut le Bel, 4, rue Blaise Pascal, 67070 Strasbourg, France. E-mail: bechinger@chimie.u-strasbg.fr*

a *Université de Strasbourg/CNRS, UMR7177, Institut de Chimie, 4, rue Blaise Pascal, 67070 Strasbourg, France*

b *Genethon, BP60, 91002 Evry, France*

c *CNRS UMR 8151, Inserm U1022, Université René Descartes, Chimie-Paristech, Paris, France*

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Abbreviations used: *ITC, isothermal titration calorimetry; MAS, magic angle spinning; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; REDOR, rotational echo double resonance.*

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Biography

Burkhard Bechinger obtained his PhD in 1989 at the Biocenter of the University of Basel in Switzerland on the investigation of electrostatic interactions within lipid bilayers using membrane biophysical approaches. He deepened his knowledge in using solid-state NMR spectroscopy for the investigation of membrane-associated polypeptides during his postdoctoral stay at the University of Pennsylvania (1990 – 1993), and started his own research group in 1993 at the Max Planck Institute of Molecular Physiology, Dortmund, Germany, and served as the head of an independent junior research group at the MPI of Biochemistry in Martinsried, Germany (1995 – 2001). Since 2001 he is full professor at the Chemistry Department of the University of Strasbourg, France, where his team designs and studies peptides with different biological activities as well as membrane proteins using NMR spectroscopy and a variety of other biophysical approaches.

Verica Vidovic graduated in 2006 from the University of Strasbourg, France, as a Master in Biology and Chemistry. She joined the laboratory of membrane biophysics and NMR in 2006 as a PhD student under the supervision of Prof. Bechinger at the same University. Her research is focused on the production and isotope labeling of antibacterial peptides in *Escherichia coli* overexpression systems. She also studies the interactions between DNA and peptides by solid-state NMR and other biophysical approaches.

Philippe Bertani was born in 1972. He obtained his PhD in physical chemistry under the supervision of Dr Jérôme Hirschinger at the University of Strasbourg in 2001, working on distance measurement by solid-state NMR. After a postdoctoral stay at the CEN in Saclay, France, performing solid-state NMR applications on calcium silicate hydrates, in 2002, he joined the group of Burkhard Bechinger in Strasbourg, where his research focus is on solid-state NMR of membrane proteins and peptides.

Antoine Kichler was born in 1966. In 1994, he obtained his PhD in pharmacochemistry (University Louis Pasteur in Strasbourg, France). He did his postdoctoral studies at the Institute of Molecular Pathology in Vienna (Austria) and then at the Centre de Biophysique Moléculaire in Orléans (France). Since 1997, he is a group leader at Genethon in Evry, France. His research interests lie in nonviral gene delivery and in the development of therapeutic approaches for muscular disorders.

Table 1. Sequence of cationic peptides used in transfection experiments

Peptide	Sequence	References
KALA	WEAKLAKALAKALAKHLAKALAKA LKACEA	[6]
Hel 11-7	KLLKLLLKLWKKLLKLLK	[8]
MPG	GALFLGFLGAAGSTMGAWSQPKSKRKV	[9]
ppTG20	GLFRALLRLLRSLWRLLLRA	[10]
Vpr52-96	DTWTGVEALIRILQQLLFIHFRIGCRHSRIG IIQQRRTRNGASKS	[11]
LAH4	KKALLALALHHLAHLALHLALALKKA	[12]

of the influenza virus hemagglutinin, such as GLF GAI AGFI ENGW EGMI DGWYG [5]. The explanation for the enhanced transfection is that the fusogenic peptides liberate the polylysine/DNA complexes from being trapped in the endo-lysosomal compartment. By adding pH-dependent fusogenic peptides, the transmembrane passage of the plasmid into the cytosol is facilitated after partial disruption of the membrane of the acidic vesicles. Notably, the permeabilization step is related to the protonation of the side chain carboxyl groups of the peptides, which induces a conformational change (from random coil to α-helix).

Taken together, these results indicate that addition of anionic fusogenic peptides is one of the possible strategies to promote endosomal escape. Ideally, however, it would be interesting to develop vehicles that become membrane permeabilizing themselves once the pH drops. The first such multifunctional peptide was KALA (Table 1), which can bind DNA, destabilize membranes and mediate significant gene delivery [6]. By replacing the negatively charged Glu residues of GALA (WEAAL AEALA EALAE HLAEA LAEAL EALAA) by cationic Lys residues, a synthetic amphipathic peptide was designed to interact with lipid bilayers at acidic pH (Table 1). In a similar manner, the negative amino acids of the anionic fusogenic peptide JTS-1 were replaced by Arg residues in the designer peptide ppTG20 [7]. Other amphiphilic peptides such Hel 11-7 also have an *ab initio* designed sequence [8]. Another strategy to develop multifunctional peptides for gene delivery consists in using viral amino acid sequences. MPG and Vpr52-96 belong to this second class of peptides (Table 1).

The Proton Sponge Trick

Polyethylenimines (PEI) belong to the most efficient cationic transfection agents developed so far [13]. It was shown that this high efficiency relies on the capacity of the polymer to buffer the endosomes, which provokes a massive proton accumulation followed by passive chloride influx. These events cause osmotic swelling and subsequent endosome disruption, thus permitting the escape of endocytosed materials. This has been called the proton sponge effect, a concept that has been used by Midoux and colleagues to engineer a polylysine with high transfection capacities. Indeed, by coupling His residues onto the polymer, they generated a PEI-like conjugate as the imidazole group has a pKa of about 6 [14]. On the basis of these results, we investigated whether it is possible to design peptides with similar properties than PEI or polylysine-His.

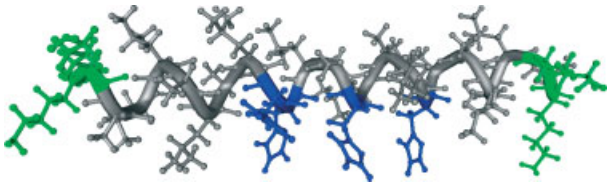

Figure 1. Schematic view of LAH4. The four Lys residues and the four His are shown as ball models, with the Lys residues at the termini (green) and the His residues in the center of the amphipathic helix.

Design and Evaluation of His-Rich Cationic Amphipathic Peptides

During the recent years, we have developed a number of peptides that are composed of Ala and Leu residues with two Lys residues at each terminus [12,15]. The Leu–Ala core of these sequences is interrupted by four His, which in an α-helical conformation all line up on one face of the helix, thereby creating an amphipathic structure [15,16] (Figure 1).

These peptides not only exhibit pronounced antimicrobial activities comparable to those of, e.g. magainins [17–20], but also potent DNA transfection activities [12]. Furthermore, we recently showed that they are potent siRNA delivery vehicles as their efficiency is higher than that of well-established compounds such as lipofectamine, dioleoyl trimethylammonium propane (DOTAP) and polyethylenimine [21]. Here, we report on a number of biophysical investigations aimed at a better understanding of the transfection activities of these peptides as well as modifications of the initial LAH4 sequence that provide improved transfection activities [18,22–24]. The simultaneous antimicrobial and DNA transfection activities of these peptides, which are both retained in the complexes with DNA, are of potential interest for multimodal applications [12], for example, in hereditary diseases such as cystic fibrosis, where *Pseudomonas aeruginosa* infections are a major health issue.

Gel shift and biochemical experiments indicate that a large peptide–DNA complex forms and enters the cells via an endosomal pathway [12]. Efficient transfection requires that the information carried by the nucleic acids is made available at their cytoplasmic or nuclear destination, respectively, and as a consequence, complexes that are too tight will probably not be efficient in such biological assays. Therefore, we have investigated the association/dissociation equilibrium of the LAH4–DNA transfection complexes as a function of pH in considerable detail by ITC [25]. These studies show that under saturating conditions and at pH 7.5, one peptide is associated with about 2 bp of DNA. Association occurs in the micromolar range and, according to the thermodynamic signature of the ITC experiment, is driven by electrostatic interactions. As the His side chains are probably uncharged at neutral conditions [15], the data suggest that the peptide interacts with its Lys termini with the negatively charged phosphates of the DNA. Notably, with two Lys side chains at each terminus the peptide is capable of interconnecting distant parts of the extended DNA molecule, as well as different DNA strands, thereby leading to the observed condensation of this polymer.

At pH $<$6, such as in the endosomal compartment, the His side chains become positively charged, increasing the overall charge to 8–9 and releasing about half of the peptides from the transfection complex [25]. At the same time, the thermodynamic signature of the ITC experiments is indicative that hydrophobic and van der Waals contacts also play a role in addition to electrostatic interactions. These observations suggest that considerable rearrangements occur within the complex. More importantly, however, a large fraction of the peptides are released and are readily available to interact with the endosomal membranes.

In this context, it is interesting to note that the interactions of LAH4 peptides with membranes have already been studied in considerable detail. The peptide adopts a predominantly α-helical conformation in the presence of membranes [16,17] and a pH-dependent topology, being transmembrane at pH 7 but oriented along the surface at pH $<$6 [15]. The transition is reversible but slow on the 10^{-4} s time scale [15,26]. Notably, the pH-dependent transition of the peptide from surface-oriented to transmembrane is paralleled by a shift of the helical regions of the peptide from a more *C*-terminal localization (encompassing residues 9–24 at pH 4.1) to a more *N*-terminal localization (residues 4–21 at pH 7.8). At intermediate pH, two shorter helices are interrupted by a hinge region formed by residues 10–13 [16].

In order to design more efficient analogs, we have started to investigate in detail not only the composition but also the size, structure and intermolecular contact of and within the LAH4–DNA transfection complexes, and this work is still ongoing in our laboratories. Zeta-sizer measurements indicate that the complexes are characterized by a hydrodynamic diameter in the 100 nm to micrometer scale depending on the environmental conditions. Whereas larger complexes are formed in the presence of salt, the positive surface charge density of the complexes probably causes electrostatic repulsion and thereby limits the aggregate size in the presence of 5% glucose [27]. In both cases, zeta-sizer measurements indicate an overall small positive surface charge density of these aggregates. When the complexes were investigated by proton-decoupled ^{13}C MAS solid-state NMR spectroscopy, the Ala- β-carbons resonate at 15.6 ppm and the carbonyls at about 176 ppm, indicating that in the pure peptide powder as well as in the transfection complex the sequence adopts an α-helical conformation [25].

In the next step, a peptide sample being uniformly labeled with ^{15}N and/or ^{13}C was prepared in order to test by REDOR solid-state NMR spectroscopy which of the side chains exhibit close proximities ($<$8 Å) to the ^{31}P nuclei of the DNA. Our first attempts to identify such distances by using compounds prepared by solid-phase peptide synthesis with a specific ^{13}C label at the β-positions of Ala-6 and Ala-13 indicated that these residues do not reside in the proximity of the DNA phosphates, and it was therefore necessary to develop a bacterial overexpression system that permitted the production of peptides uniformly labelled with stable isotopes at reasonable costs [28]. In view of the antimicrobial activity of the peptide, this required a rather tight expression system and the neutralization of the product within inclusion bodies. Although, the bacterial over-expression and purification resulted in the addition of a Pro residue at the *N*-terminus; this P-LAH4 peptide exhibits comparable antimicrobial and transfection activities when compared to the LAH4 peptide prepared by solid-phase peptide synthesis.

When the REDOR effect was measured using uniformly labeled compounds dipolar couplings were detected between the Lys side chain amines and the phosphate groups but not for any of the other ^{15}N or ^{13}C side chain resonances (Figure 2(A)). The REDOR dipolar dephasing curve of LAH4 in the complex with DNA is shown in Figure 2(B) for the resonance corresponding to the four ε-^{15}N-Lys atoms. The distance is in the range of 5 Å and to our knowledge this is the first time that the molecular interactions within a transfection complex have been analyzed with accuracy.

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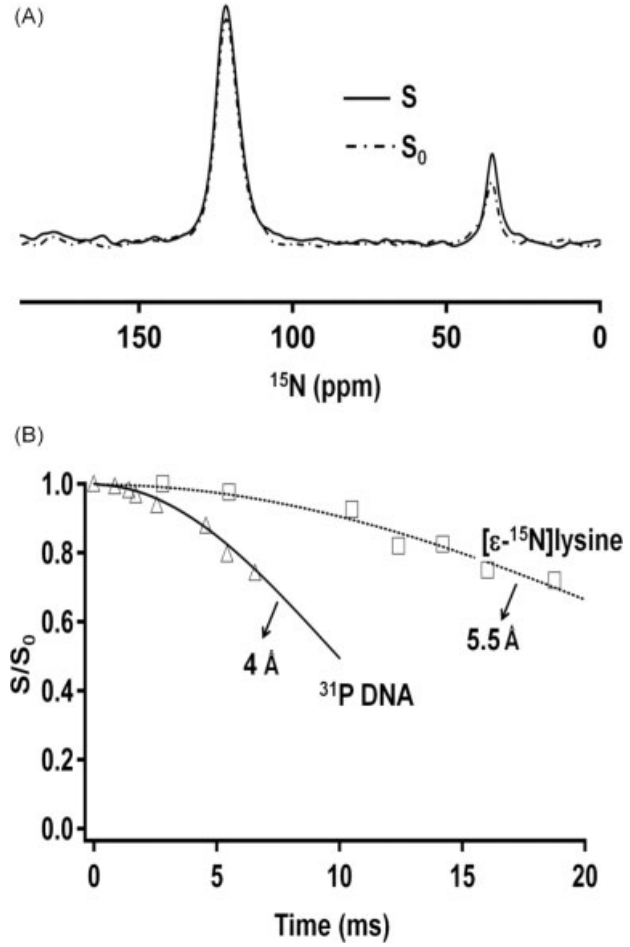

Figure 2. (A) ^{15}N $\{^{31}P\}$ REDOR spectra of uniformly ^{15}N labeled P-LAH4 peptide complexed with salmon sperm DNA, after 19.1 ms of dipolar evolution time (at a MAS spinning speed of 22 kHz). The full-echo spectrum where the dipolar couplings are averaged (solid line) and the spectrum with reduced signal intensities due to the dipolar couplings (dotted line) are shown. The spectra are the result of the accumulation of 2048 scans. (B) ^{15}N $\{^{31}P\}$ and ^{31}P $\{^{15}N\}$ REDOR dephasing curves as a function of dipolar evolution time, for the uniformly ^{15}N labeled P-LAH4 peptide complexed with salmon sperm DNA. The observed experimental dephasing (square) of the ^{15}N signal arising from the four Lys side chains (ε position) and the *N*-terminus has been fitted to the decay curve expected from a single ^{15}N–^{31}P pair at 5.5 Å interatomic separation (dotted line). The experimental dephasing of the ^{31}P spectrum from the DNA of the same sample (triangles) is consistent with a single ^{15}N–^{31}P distance of 4 Å (solid line). The difference in the apparent distance probably arises from multiple dipolar interactions involving one or two Lys residues at each terminus and several phosphates along the DNA polymer.

With this data, peptides were again prepared by chemical peptide synthesis as this method is ideally suited to prepare sequences labeled at only one selected site. Notably, the work is ongoing as triangulation based on additional distances should allow the development of a detailed structural model (Figure 3).

The data suggest that electrostatic interactions play an important role in the condensation, dimensions and composition of the DNA–LAH4 complexes. Interestingly, reversible flocculation phenomena of lipid vesicles driven by electrostatic interactions have also been observed when the cationic LAH4 peptide was added to anionic lipid vesicles. Whereas the POPC/POPS vesicles remained in suspension in the absence or in the presence of excess amounts of LAH4, peptide-mediated aggregation of these vesicles was observed when the surface charge density of the membranes approached neutrality [29]. When the ratio of anionic POPS was low, the aggregation was reversible but fusion occurred at PS concentrations exceeding 25%. Similar activities have been observed for other cationic peptides [30,31], but presently it is not clear if such flocculation, membrane aggregation and fusion activities play a role during the antimicrobial or transfection activities of these peptides.

Conclusions

The biophysical studies on LAH4–DNA nanocomplexes have revealed three fundamental requirements that should be included into the design of new compounds working along related mechanisms [25]. First, complex formation by electrostatic interactions is easily accomplished by cationic functional groups. Second, the release of the molecules from the complex upon acidification requires titration of some residues (such as His residues), concomitant with a significant modification of the electrostatic interactions when going from neutral to acidic pH. Third, the liberated molecules should exert membrane lytic activities in the endosomal context. Notably, this 'efficient' release from the endosomal compartment seems to differentiate LAH4 from other cell-penetrating peptides that efficiently associate with cells but where endosomal escape turns out to be a limiting factor [32].

Although, the above points define some fundamental rules as to the design of novel sequences, modifications of the LAH4 sequence seem to indicate that they are necessary but not sufficient and that other not so well-understood properties of the peptides also influence transfection efficiency. On the one hand, the above criteria explain why, for example, LAH4 peptides carrying additional Lys residues work less well as these permanent charges probably reduce the amount of peptides liberated upon endosomal acidification [27]. On the other hand, it is less obvious why conservative replacements of amino acids at a single site, shortening the peptide by only two residues, or the preparation of the all-D enantiomer all result in significantly decreased DNA transfection activities [24,27]. Interestingly, increased activities could be obtained in the presence of serum after amino acid rearrangements of the initial LAH4 sequence [24]. Furthermore, an interesting correlation between transfection activities, the disruption of membrane order and the angle suspended by the hydrophobic residues when viewed along the helix long axis (Edmundson helical wheel projection) could be identified [22]. These observations suggest that other steps during transfection such as transport to and into the nucleus, proteolytic activities outside and inside the cells or interactions with other biomolecules are also important. Notably, the entry into the nucleus limits gene expression but not siRNA activities, thereby explaining why efficient endosomal escape makes LAH4 such a potent siRNA transfectant [21] albeit very little biophysical information currently exists on the siRNA–LAH4 complex.

Perspectives

Although efficient for DNA and siRNA transfection when compared to other nonviral vectors, it will be necessary to further improve the activity of the LAH4 family in order for these peptides to become a vector system for human gene therapy. To achieve

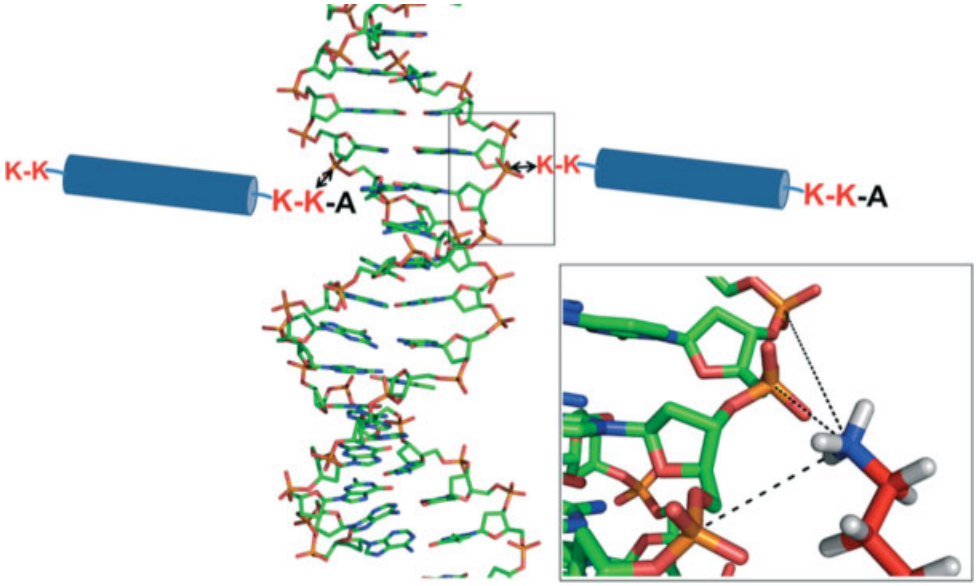

Figure 3. Schematic model illustrating the close proximity of the terminal Lys residues and the phosphates from the DNA. The central helical domain is represented as a cylinder whereas the insert illustrates the interactions of the ^{15}N of one Lys side chain with the ^{31}P atoms of the DNA. The dense coverage of DNA with peptide (about 1 peptide/2 bp), in combination with the absence of close contacts between the Ala, Leu or His residues and the phosphates suggest a head-on rather than a helix-into-groove packing.

this goal, it will require both a deeper understanding of how to control the size and composition of the nanoscale complexes, how the peptides function as well as the evaluation of additional modifications of the sequences.

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