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Review

Peptide-nucleic acid nanostructures for transfection

Burkhard Bechinger

Université de Strasbourg/CNRS, UMR7177, Institut de Chimie, 4, rue Blaise Pascal, F-67070 Strasbourg, France

e-mail: bechinge@unistra.fr

Abstract

To use nucleic acids in biomedical research and medical applications, these highly hydrophilic macromolecules have to be transported through the organism, targeted to specific cell surfaces, and have to cross cellular barriers. To this end, nanosized transfection complexes have been designed and several of them have been successfully tested. Here, the different steps of the transfection process and the particular optimization protocols are reviewed, including the physicochemical properties of such vectors (size, charge, composition), protection in serum, cellular uptake, endosomal escape, and intracellular targeting. The transfection process has been subdivided into separate steps and here special emphasis is given to peptides that have been designed to optimize these steps individually. Finally, complex devices encompassing a multitude of beneficial functionalities for transfection have been developed.

Keywords: cell-penetrating peptides; gene therapy; nanovectors; siRNA; transcription.

Introduction

The therapeutic potential of nucleic acids is well recognized and biomedical applications such as gene therapy or medical treatments using short oligonucleotide sequences are subject of intense research (1, 2). These molecules have the potential to be used in a specific manner, with high selectivity and potency. However, for medical applications, the nucleic acids have to be delivered to the correct target tissues. Furthermore, small interfering RNA (siRNA), antisense nucleotides and DNA have to cross the cellular membrane barriers to accumulate in the cytoplasm or nucleus, respectively (3).

Whereas the nucleic acids alone are much too hydrophilic for easy membrane passage, nature has overcome this problem with specialized machineries, and one approach consists in exploiting the properties of viruses such as adeno-associated virus (AAV). This virus corresponds to a supramolecular assembly of DNA and proteins of about 22 nm in size (Figure 1A). During infections, the capsid of AAV is released and attaches to the nuclear pore to transfer the genomic DNA directly into the nucleus (4, 5). Influenza viruses use a different pathway where the low endosomal pH induces a conformational change in the hemagglutinin (HA) protein to expose a domain, which results in the fusion of endosomal and viral membranes. Other viruses release their RNA directly into the cytosol (4, 6, 7). Indeed, viral delivery is currently the most widely used approach, including in clinical trials. However, these viral vectors also exhibit considerable side effects and in addition some are limited, for example, in the maximal size of DNA fragments (typically <5 kbp) that they can carry (8).

Therefore, in parallel, 'non-viral gene delivery systems' have been designed and developed and many of these are based on synthetic cationic molecules (Figure 1B,C), where it is considered that these interact and condense the DNA in a manner that is related to the packing by histones (9–11). To this end, a great variety of molecules have been prepared, ranging from synthetic lipids to polymers, dendrimers, nanoparticles, and peptides (8, 12). Indeed, cationic liposomes have already been introduced in 1987 and in combination with nucleic acids form the so-called lipoplexes (13). Inclusion of neutral helper lipids has been shown to enhance their transfection activity, which by its fusogeneic activities helps in the release of the cargo from the endosomes (14).

Although most cationic compounds are able to compact DNA, this turns out to be a necessity but insufficient for efficient transfection into eukaryotic cells. Many additional requirements need to be fulfilled such as protecting the DNA against enzymatic degradation, the cellular uptake of the complexes, and their delivery to the correct target organelles, which involves endosomal escape of the plasmid and if delivery of genetic information is desired also the entry into the nucleus (recently reviewed in refs. 3, 8, 15). Whereas many of the above considerations have been defined in the context of gene delivery, short RNA polymers have more recently become the focus of attention as they bear a multitude of potential biomedical applications. Many of the general considerations apply equally well also for RNA transport when at the same time important details vary, such as the size of the resulting complexes and differences in their final destination, being the cytoplasm rather than the nucleus.

Many of the non-viral vectors complex with the DNA in a manner to form nanosized to microsized particles, or they are designed in such a manner to assemble in well-defined shapes and size (Figure 1B,C). In view of their less pronounced side effects, they seem safer but for the time being they are in general also less efficient than the viruses (12). Therefore, much effort is dedicated to improve their efficiency and their

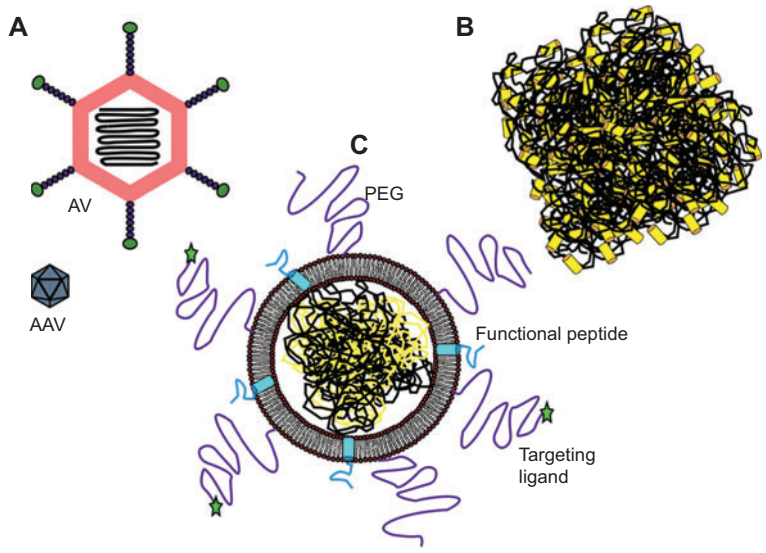

Figure 1 Structures of viral and non-viral vectors.
Schematic illustrations of (A) adenovirus and adeno-associated virus. Adenoviruses (AV) are made of typically 12 different protein constituents that form an icosahedral capsid (red) and spikes (violet and green). These encapsulate 25–45 kbp of double-stranded linear DNA (shown as black line). The diameter of these viruses is 80–95 nm (129). The adeno-associated viruses are satellite particles (gray, AAV), are 22 nm in size and of icosahedral geometry. They encompass three different capsid proteins and 4.7 kb of single-stranded DNA. As they need helper viruses to duplicate, they are not pathogenic (130, 131). (B) The LAH4-based transfection complexes are typically 100 nm to micrometers in size and carry a slightly positive zeta-potential (107). The complexes efficiently transfect small interfering RNA as well as large plasmids (70). The peptides are shown in yellow and the nucleic acids in black. A limited amount of structural data exists on the overall and internal structure of the complexes (cf. text and Figure 2 for details). (C) Multifunctional envelope-type nanodevice (MEND) simulating the structure of enveloped viruses. A core of DNA/cationic polymer (e.g., polylysine) of about 100 nm in diameter is prepared and surrounded by an overall negatively charged lipid bilayer. The latter is decorated with polyethyleneglycol (violet), functional peptides (blue), and targeting ligands (green stars). MENDs are typically 300 nm in diameter and exhibit a zeta-potential of -40 mV (132).

properties. In particular, major issues that need to be considered are the stability of the complexes in biological environments such as the bloodstream, them reaching the target and avoiding non-target sites, efficient endocytosis, but also endosomal escape and for some applications getting the genetic information into the nucleus (Figure 2). Although transfection has become a major tool that is by now routinely applied in fundamental and applied research, major improvements are still required for *in vivo* and medical applications.

In the context of this review, the focus is on how peptides form delivery complexes with nucleic acids and how they can be used to overcome the existing limitations. Peptides bear a number of advantages when future biomedical applications are taken into consideration such as the ease of product identification and quality control as well as the possibility of well-established production processes at low and large scale, which are all important properties. Furthermore, their characteristics can easily be manipulated by changing the peptide sequence or by inclusion of non-natural amino acid residues. Indeed, when combining cationic peptides and nucleic acids, many of them spontaneously self-associate into complexes by electrostatic interactions (Figure 1B), and some of these sequences have been shown to exhibit excellent capacities to deliver nucleic acids into cells (3, 16).

Much research in the nanobiosciences is concerned with overcoming the drawbacks of non-viral delivery systems and to systematically design mechanisms for targeted drug release, to control biodistribution, and to maximize the therapeutic efficacy while avoiding unwanted side effects [reviewed in ref. (17)]. In this context, tuning the size of the nanostructures becomes an important parameter that influences their ability

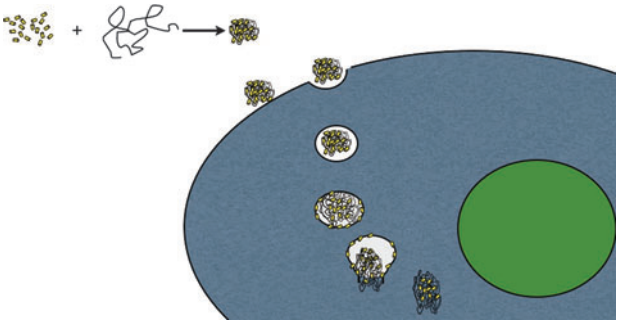

Figure 2 The transfection of DNA with the multifunctional LAH4 peptide is schematically illustrated.
Upon mixing under physiological conditions, LAH4 peptides condense plasmid DNA. The resulting complexes are taken up by endosomes and upon acidification of this organelle about half of the peptides are released from the complex and many are available for membrane interactions. The high concentration of amphipathic peptide probably results in membrane lysis and the release of contents into the cytoplasm. This self-promoted uptake of nucleic acid/peptide complexes has been used to efficiently transfect small interfering RNA but also a 7.6 kb plasmid expressing luciferase into eukaryotic cells (70).

to cross membranes but also slows down their clearance from the body (18, 19). Whereas smaller particles have lower cargo capacity, they bear at the same time the advantage of better dissolution rates (20). Therefore, even within the nanoscale, the correct choice of size, shape, and surface properties are important for bioavailability and blood circulation time and the question arises what is the optimal size of such a complex (21–23). On the one hand, to reach the respective target cells, the complexes need to circulate through capillaries and the size should therefore not exceed 70 nm (24). On the other hand, the somewhat lager systems (<200 nm) have longer half-lives in the body (21). Whereas nanometer-sized complexes are also taken up by endocytosis (25), it has been shown that particles >200 nm are quickly eliminated by phagocytes (26). Notably, liposomes with an average diameter of 100–200 nm have been shown to efficiently accumulate at tumor tissues (27).

In another approach, the shape of the nanocomplexes has been controlled through β-sheet scaffolds, which have been used for the assembly of siRNA transfection complexes (28). In this instance, hydrophilic segments prevent amyloid formation, cationic functional groups have been added for siRNA binding, and addition of glucose renders the ribbon charge-neutral. Shaping delivery vehicles for an order of magnitude-improved activity has also been achieved in the form of carbon nanoneedles made of carbon nanotubes with functionalized surfaces (29). Furthermore, nanosized micelles of triblock copolymers have been shown to transport into cells siRNA together with the anticancer drug paclitaxel or other cargos (30).

The rational design of nanoparticles is based on combining modules of selected properties and in such a manner drug delivery systems have been manufactured for specific applications. The approach permits to modulate all of the hydrophilicity and hence solubility, the size and specificity of targeting groups, thereby affecting the biodistribution, biocompatibility, biodegradation, and interactions between the drug and the carrier (19). Functionalization of the surface of the nanostructure can be used for specific receptor or surface interactions (31, 32), including the selection of intracellular targets (33, 34). In this context, it is interesting to note that the surface charge of the particle determines if they remain in lysosomes or are targeted to the cytoplasm and mitochondria (33). With these goals in mind, peptides are designed and/or included into complex delivery systems to specifically overcome one or several of the hurdles that hamper efficient nucleic acid delivery.

Augmenting the stability in serum

The stability of transfection complexes has been improved by coverage of the surface with polyethyleneglycol (PEG). In the case of liposomes, this modification leads to the so-called stealth liposomes, which successfully evade the immune system (35). The PEG coverage prolongs blood circulation, reduces non-specific interactions with the reticuloendothelial system, and improves size uniformity, but it also decreases cellular uptake (15). Therefore, a number of additional alterations have been introduced, such as chemical modifications of the PEG, or addition of TAT peptides (Table 1) to liposomes, which in some instances but not in a predictable general manner, have improved transfection activities (15). Linking TAT to PEG-poly(ethyleneimine) (PEI) increases *in vivo* but not *in vitro* activities (36). Linking phosphatidylethanolamine to PEG and TAT improved the activities of exon skipping nucleotides *in vivo* (37). PEG is also used in complex edifices

Table 1 Sequences of cationic peptides as well as nuclear targeting sequences mentioned in this review (including selected references, cf. text for details).

Cationic peptides		
HA2 (1–23)	GLFGA IAGFI ENGWE GMIDG WYG	(63)
INF7	GLFEA IEGFI ENGWE GMIDG WYG	(65)
GALA	WEAAL AEALA EALAE HLAEA LAEAL EALAA	(59)
KALA	WEAKL AKALA KALAK HLAKA LAKAL KACEA	(72)
Hel 11-7	KLLKL LLKLW KKLLK LLK	(75)
MPG	GALFL GFLGA AGSTM GAWSQ PKSKR KV	(125)
JTS-1	GLEEA LLFLL ESLWE LLLEA	(63, 65)
ppTG20	GLFRA LLRLL RSLWR LLLRA	(126)
Vpr52–96	DTWTG VEALI RILQQ LLFIH FRIGC RHSRI GIIQQ RRTRN GASKS	(127)
LAH4	KKALL ALALH HLAHL ALHLA LALKK A	(70, 96)
Penetratin[a]	RQIKI WFQNR RMKWK K	(128)
TAT[b] 48–60	GRKKR RQRRR AHQ	(128)
IRQ peptide	IRQRR RR	(124)
4_3E	$(LAEL)_3$	(116)
4_6	Ac-$(LARL)_6$-NH2	(116)
Nuclear targeting sequences (examples)		(108)
SV40 large T-antigen	PKKKR KV	
Nucleoplasmin	KRPAA IKKAG QAKKK K	
HIV-1 Rev	RQARR NRRNR RRRWR	
hnRNPA1 (M9)	NQSSN FGPMK GGNFG GRSSG PYGGG GQYEA KPRNQ GGY	

[a]*Drosophila* Antennapedia. [b]Transactivator of transcription.

for nucleic acid delivery (38) or has been directly conjugated to siRNA to protect it from nucleases (39). An alternative route is to cover the transfection complexes with human serum albumin (40).

Specific interactions with the target cells and improving cellular uptake

By conjugating to the vector high-affinity ligands for selected receptors of the target cells, efficient uptake of these complexes has been achieved. Although this concept was much used in the context of lipoplexes, some of the work should be mentioned here as it provides valuable guidelines for future developments of peptide-nucleic acid nanocomplexes. For example, receptor-mediated endocytosis was observed when the ligands were growth factors or transferrin (41, 42). In a related approach, liposomes were coated with the 12-residue peptide AG73 specific for cancer cells (43), with a human immunodeficiency virus (HIV) receptor protein (44) or with antibodies (45). As endocytosis can also occur in a receptor-independent manner, many other parameters controlling the interactions between the particle and the cell are important, including the particle size and its shape or the surface charge (25, 46). For example, the uptake of an inorganic particle covered with TAT peptides was optimal when their diameter was about 200 nm (47). The addition of TAT helped in the internalization into HepG2 cells. Furthermore, cell-penetrating peptides (CPPs; also called 'protein translocation domains') enhance the cellular uptake of various cargos where one of several possible pathways is through endocytosis (15). Another positively charged vector has been introduced by complexing nucleic acids to cationic liposomes (lipoplex) with efficient complexation and transfection activities (13). The addition of polyglutamates to change the surface charge of cationic polymers made them less toxic and decreased agglutination phenomena (48). Notably, it has been shown that through absorption of plasma proteins, particles undergo charge reversal when investigated in Dulbecco's (positive surface charge) or DMEM/fetal calf serum medium (negative) (47) and such changes in physicochemcial properties of the transfection complexes and/or of the accessibility of surface structures/ligands may, in addition to enzymatic activities, be responsible for the often observed decrease of transfection activities in the presence of serum.

CPPs have also been shown to promote cellular uptake and to mediate the transport of various cargos across membranes. However, they are insufficient to achieve efficient transfection of big hydrophilic cargos such as nucleic acids by themselves. Nevertheless, in combination with other strategies, several presented in this paper, CPPs are used to stimulate cellular uptake of the complexes either by macropinocytosis or by endocytosis (15, 46). Furthermore, they confer a positive charge to the transfection complexes even if used in conjunction with anionic fusogenic lipids (49). In this context, it is notable that although poly-lysines enhance transfection, this effect is more pronounced for arginine-rich peptides (50). The poly-R complexes proved fusogenic at acidic and neutral pH, whereas the presence of poly-K only under neutral conditions indicates that they play a role beyond conferring a positive electrostatic potential to the transfection complex.

Therefore, arginine-rich CPPs such as the HIV-derived TAT peptide (residues 49–61) or oligoarginines are often found in transfection mixtures (15). For example, TAT was combined with the transfection polymer PEI (cf. below for details) and improved its activity *in vitro* but not *in vivo* (51). PEI was also used in combination with the Antennapedia CPP (Table 1) (52). Furthermore, these sequences have been included in hybrid peptides where different functionalities are combined (15), or in complex nanodevices for transfection [ref. (38); cf. below]. Notably, whereas the TAT-PEG-PEI/DNA polyplex has improved transfection activity when compared with PEI alone (53), the covalent attachment of undeca-arginine (R11) to PEI has the opposite effect (54). When TAT was mixed with cationic lipids and plasmid DNA added in a last step, good transfection efficiency was obtained (55). However, adding TAT to preformed lipoplexes works less well, indicating that the topology plays a key role. Furthermore, as the overall charge of the complex is an important parameter, an optimum exists for the ratio of peptide/transfection reagent (55).

Enhancement of endosomal escape

For transfection complexes that enter the cell through endocytosis, a critical step is their capability to escape the endosome and to deliver the cargo to the cytoplasmic environment (12, 46). Many systems have been described where nucleic acid delivery is severely hampered by the entrapment of cargo in this organelle. This important step of transfection has therefore already been the subject to a number of recent reviews (8, 15, 46, 56). It should be noted that some of the additions and modifications cited in a different context in this review may also affect the endosomal escape during the transfection process.

Early on, polylysine was used as a transfection agent; however, this homopolymer performed poorly when only mixed with DNA (57). Therefore, it was soon realized that adding fusogenic anionic peptides to the polylysine/DNA complexes much improved efficiency. In particular, the N-terminal segment of the HA-2 domain of the influenza virus HA (Table 1) as well as derivatives thereof are used to this end (58). The presence of the pH-dependent fusogenic peptides facilitate the transmembrane passage of the nucleic acids into the cytosol by partial disruption of the membrane after acidification of the vesicles, thereby preventing the entrapment of polylysine/DNA complexes in the endolysosomal compartment. 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) (58).

The designed GALA peptide (Table 1) is water soluble at neutral conditions and undergoes a random coil-to-helix transition when the pH is decreased to 5 (59). The helix conformation shows a high affinity for membranes (59) where pores encompassing about 10 peptides are formed (60, 61). Both GALA and HA2 fusogenic peptides have been used as additives to DNA transfection complexes, including lipoplexes

(59, 62–64). One may wonder how it is possible that their action is not toxic to the cell as they must also liberate a number of endosomal hydrolytic enzymes into the cytoplasm; however, for optimal activities, these enzymes require pH values below those of the cytoplasm (8).

INF7 is a modified HA2 sequence known to form pores in liposomes with an activity that is increased at low pH and better than that of the parent peptide (Table 1). It is used as an additive to cationic liposomes or polymer transfection complexes (65–67). A 50-fold increase in chinese hamster ovary (CHO) cell transfection has been observed upon addition of INF7 to a transfection mixture of PEG and a DNA-binding peptide (68). Furthermore, INF7 improved siRNA delivery of magnetic nanoparticles associated with complexes of siRNA and PEI (69).

It should be noted that the above-mentioned investigations on the role of the decrease in endosomal pH were possible by using chloroquine, a molecule that prevents endosomal acidification. On the one hand, when added during cell culture experiments, it was shown that several of the delivery systems tested need the acidic pH to trigger endosomal release (70). On the other hand, the same compound has been included to protect the cargo from endosomal degradation, and in this context it can be beneficial to transfection efficiency (12, 71). In any case, although being a useful tool for research, application of chloroquine is impractical for *in vivo* experiments (12) and using endosome-disruptive polymers and/or membranelytic peptides in the transfection systems seems a more promising approach.

Together, the cited results indicate that the addition of anionic fusogenic peptides provides a valuable strategy to promote endosomal escape. However, in an ideal world, the peptides would act as the complexation agent, help in endosomal uptake, and become themselves membrane permeabilizing in response to pH. In this context, KALA was developed to be such a multifunctional peptide for gene delivery (72). By replacing the anionic glutamines of GALA by positively charged lysines, a novel amphipathic peptide was designed to interact with lipid bilayers at acidic pH (KALA, Table 1). As a further development along this line, KALA was self cross-linked through a cystine bridge (73). Similar siRNA gene silencing activities could be achieved as with PEI; however, the peptide is somewhat less toxic. The cross-link assures more stable and smaller complexes with siRNA, which the authors of the study assign to the higher charge density (73). Furthermore, in the presence of serum, shielding of the complex with PEG improved the transfection activities (39, 73).

In a similar manner, the negative amino acids of the anionic fusogenic peptide JTS-1 were replaced by arginines to create the designer peptide ppTG20 (Table 1) (74). Other amphiphilic peptides such Hel 11-7 were designed to improve transfection activities (75), or multifunctional peptides such as MPG and Vpr52–96 were derived from viral amino acid sequences (Table 1).

An alternative strategy following the same line of ideas is to use fusogenic lipids such as DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) mixed with lipoplexes (14) to facilitate membrane fusion (76). The PE (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine) lipids are known to exhibit a tendency to adopt inverted hexagonal phases (77), in particular when the pH is lowered. This inverted hexagonal phase is known to promote fusion of lipid bilayers and as a consequence the escape of endosomal contents [mechanisms reviewed in ref. (78)]. Decorating DOPE-containing DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) lipoplexes with PEG stabilizes the lamellar phase and thereby reduces transfection efficiency (79). Transport through membranes has also been achieved by using the T-domain of diphtheria toxin (DT) (80–82), which undergoes profound conformational changes when interacting with membranes and/or when exposed to acidic pH (83).

A conceptually different strategy is followed by incorporating endosome-disrupting polymers or lipids containing either PEI or multiple imidazoles in the delivery system (84). This can be achieved by conjugation or complex formation and endosomal disruption is thought to occur through the so-called proton sponge effect (12, 56, 84). When designing such delivery systems, it is of advantage that the cationic compounds also have a tendency to bind and compact anionic polymers (12, 56), which helps overcome the size limitations of endosomal uptake (85). However, this electrostatic association has to be reversible to liberate the nucleic acids at their destination (86). Unfortunately, toxic effects have also been observed for such cationic complexes (87).

The high efficiency of polyethylenimines relies on their capacity to buffer the endosomes when the pH drops, which provokes a massive proton accumulation within the endosome followed by passive influx of counter ions such as chloride. Such an accumulation of ions causes osmotic swelling concomitant with endosome disruption, thus permitting the escape of the transfection complexes and other material. The proton sponge effect has been used to engineer other polymers with high transfection capacities. Indeed, by coupling histidines onto a polylysine, they generated a conjugate simulating the effects of PEI, where the imidazole group has a pKa of about 6 and changes its properties upon endosomal acidification (88).

Furthermore, combinations of TAT peptides with polyhistidines were tested (89). Although these were not as efficient in transfection as PEI, they were considerably less toxic. Another combination investigated is the mixture of TAT and PEI (90). It should be noted that the proton sponge effect may not be generally applicable as amphipathic, fusogenic peptides much enhance the endosomal release of some polyplexes tested, suggesting that endosomal release remains an issue despite the presence of the polymer (66). However, the surface decoration with PEI has also shown advantages during viral transfection experiments and it is thought that in such circumstances, the PEI has beneficial effects by protecting the particles from serum (91).

Furthermore, hybrid sequences encompassing cell-penetrating and fusogenic peptides have also been found to exhibit favorable properties for the transfer of some polypeptides (92, 93) and were also tested for peptide-nucleic acids (94) and siRNA cargos (95).

On the basis of the ensemble of ideas presented thus far, a new family of peptides has been developed (named LAH4, Table 1). These are composed of alanines and leucines with two lysines at each terminus and four histidines in the central core region (96, 97), thereby combining within a short amino acid sequence the DNA complexation of cationic residues with a potent endosomal escape mechanism (70). Table 1 shows the LAH4 sequence, which in the presence of membranes or when associated as an oligomer form amphipathic helical structures where all four histidines line up on one side of the helix (96, 97).

The LAH4 designer peptides exhibit potent antimicrobial activities comparable to those of, e.g., magainins (98–101), and in addition they have proven valuable for the transfection of DNA (70) and siRNA (102). In fact, delivery of the latter is more efficient than that of widely used and tested compounds such as Lipofectamine (Invitrogen, Saint-Aubin, France), DOTAP, and polyethylenimine (Sigma-Aldrich) (102). Interestingly, the antimicrobial activities of LAH4 peptides are retained in the complexes with DNA and are therefore of potential interest for multimodal applications, especially in hereditary diseases such as cystic fibrosis, where *Pseudomonas aeruginosa* infections are a major threat to the patients (70). Furthermore, they have very recently been used to transport polypeptide-based vaccines adjuvanted with Toll-like receptor 9 agonist CpG oligonucleotides (103).

A series of biochemical and biophysical investigations was performed, which allows for a better understanding of the structural requirements underlying good transfection activities and the design of even more efficient LAH4 sequences (99, 104–106). First, gel shift and biochemical experiments indicate that a large peptide-DNA complex forms and enters the cells through an endosomal pathway (Figure 2) (70). In the next step, the association of the LAH4-DNA transfection complexes was investigated as a function of pH using isothermal titration calorimetry (ITC). It is observed that at pH 7.5, up to one peptide is associated per 2 bp of DNA (86). Association occurs in the micromolar range and, according to the thermodynamic signature of the ITC experiment, is driven by electrostatic interactions. The data thereby suggest that the peptide interacts with its lysines with the negatively charged phosphates of the DNA. With two lysines at each terminus, the peptide is thereby able to interconnect and condense distant parts of the extended DNA polymer. The data also show that complex formation is reversible in accordance with the necessity of making available the information stored on the nucleic acids at their cytoplasmic or nuclear destination, respectively.

In the endosomal compartment, the pH drops and the histidine side chains become positively charged (96), thereby increasing the overall charge to 8 or 9. As a consequence, about half of the peptides are released from the transfection complex (86). At acidic pH, the thermodynamic signature of the ITC experiments is indicative that interactions occur through a combination of hydrophobic, van der Waals, and electrostatic interactions suggesting considerable structural rearrangements within the complex. In addition, a large fraction of the peptides is released and readily available to interact with and disrupt the endosomal membranes.

In this context, it is interesting to note that in membranes, the peptides adopt predominantly α-helical conformations (97, 98). Furthermore, a pH-dependent topology has been observed using solid-state nuclear magnetic resonance (NMR) and attenuated total reflection Fourier transform infrared spectroscopies, being transmembrane at pH 7 but oriented along the surface at pH <6 (96). Notably, the pH-dependent transition of the peptide from surface-oriented to transmembrane is paralleled by a conformational shift of the helical regions of the peptide (97).

Zeta-sizer measurements indicate that the complexes are characterized by a positive surface charge density and hydrodynamic diameters in the 100 nm (Figure 1B) to micrometer range where the size can be controlled by the environmental conditions (107). Proton-decoupled ^{13}C magic angle spinning solid-state NMR spectroscopy shows that the peptide adopts an α-helical conformation also in the transfection complex (86). Furthermore, rotational echo double resonance solid-state NMR spectroscopy shows that the DNA phosphates are in proximity of the lysine side-chain amines, where a distance of about 5 Å is measured; however, no such contacts were detected for any of the other ^{15}N or ^{13}C side-chain resonances in agreement with the notion that electrostatic interactions are a major driving force of complex formation and DNA condensation (3).

Strategies to augment delivery to intracellular compartments

By incorporating a nuclear localization sequence (NLS, Table 1), enhanced transport into this organelle can be achieved (108, 109). Therefore, when NLS-PM10-TAT is administered in conjunction with HA2-TAT, an enhanced cytostatic effect by the 10-residue peptide PM10 is observed (34). An elegant approach was chosen by Midoux and coworkers who incorporated a DNA segment that is recognized by the nuclear factor κB transcription factor. Under well-chosen conditions and through this designed interaction, this protein shuttles between the cytoplasm and the nucleus and increases the nuclear import of plasmid DNA and the concomitant expression of a reporter gene (110). The possibility of targeting the mitochondria has also been considered [reviewed in ref. (111)].

Building complex edifices with different functionalities

To refine the transfection process, a stearoyl-block peptide containing arginines and histidines for nucleic acid binding and endosomal escape has been presented, where cysteines allow for interpeptide cross-linking (112). Cystine bridges have also been used to create reduction-sensitive polymers. The Cys-Cys bridges are stable in circulation but rapidly degrade in intracellular compartments. Therefore, they can be applied for controlled cytoplasmic delivery in the context of a wide variety of systems (113, 114).

Connecting the sequences into hybrid peptides prevents separation into different compartments (115). In a related manner, adding the designer peptide 4_3E, which forms an amphipathic α-helix at acidic pH, helps transfect complexes of DNA with the cationic 4_6 CPP (Table 1) (116). Similarly, HA2-p53-R11 augments the effect of p53 to induce apoptosis from <10% with p52-R11 alone to 50% (117).

Other systems that combine surface recognition and endosomal escape include a construct of three functional domains namely an antibody to confer target cell specificity – the DT-T domain to facilitate endosomal escape – and a DNA-binding domain derived from the yeast GAL4 protein. To this protein-DNA complex, polylysine was added to achieve an overall positive charge. The complex built resulted in the delivery of a reporter gene that was 25 times improved when compared with polylysine alone (95). Furthermore, the DT-T domain was conjugated to streptavidin and mixed with PEI polyplexes to show an order of magnitude improved transfection efficiency for a luciferase gene when compared with PEI alone (58). Notably, in these constructs, the details of the conjugation have been shown important.

Third, a protein consisting of four tandem repeats of a histone DNA condensing domain (H2A), the HER2 targeting motif, GALA, and a cathepsin D substrate to cleave the targeting motif from substrate was genetically engineered. The cleavage site was inserted to ensure better endosomal release. In this construct, GALA has been shown to significantly enhance transfection activity (118).

In the context of liposomal delivery systems, GALA-cholesteryl and transferrin have been added (119). The presence of transferrin enhances uptake, suggesting that a high surface concentration is required. Furthermore, GALA was used in combination with octaarginine to cover liposomes with siRNA (120). The negatively charged GALA was combined with the cationic Lipofectamine to ensure accumulation of the peptide to the cell surface, which works well also in the presence of serum (121). Whereas endocytosis requires Lipofectamine, endosomal escape is mediated by GALA. These data suggest that the combination of cell surface recognition and accumulation with efficient endosomal release is important.

Finally, Harashima and co-workers have assembled the complex layer-by-layer edifice for nucleic acid transfection shown in Figure 1C (38). First of all, the multifunctional envelope-type nanodevice (MEND) encapsulates condensed plasmid DNA, siRNA, protein or else inside a liposomal coat (Figure 1C). The lipids in MEND are the fusogenic DOPE as well as anionic lipids [phosphatidic acid or cholesteryl hemisuccinate (CHEMS)] enhancing endosomal escape. In addition, cationic peptides such as stearylated octaarginine (R8) are needed to impart a positive charge for cellular uptake (15). The resulting core-shell nanoparticle is functionalized by the attachment of PEG as a protective layer and receptor-targeting ligands such as transferrin (38). This combination assures longer blood circulation times while increasing association with cells. The design of MENDs are further refined by linking the PEG by a substrate peptide for metalloproteases, which is cleaved enzymatically in particular when associated with tumor cells (122). Combining this modification with the addition of fusogenic membrane peptides such as GALA resulted in synergistic enhancements of transfection activities (123). The MEND particles are not cytotoxic and more efficient than adenovirus in transfecting HeLa and A549 cells (38). The system made of DOPE/CHEMS/eggPC and R8 has been tested in the delivery of DNA, oligonucleotides, or siRNA as well as for the immunization with encapsulated antigen (38). Furthermore, R8 has been replaced with other R-rich CPPs (123).

Outlook

With progress picking up and more and more research teams working in the field, it can be expected that important developments in using peptide-nucleic acid nanocomplexes *in vivo* will be made, and on a longer-term valuable feedback will also come from clinical trials. As good knowledge and many tools are available to create and shape peptide-nucleic transfection complexes *in vitro*, a better understanding of their *in vivo* transfection capacity will be an important step in better defining the requirements that need to be implemented. The structural and biophysical characterization of the best complexes will not only be of academic interest but will also allow the directed design of specific properties in a highly efficient manner.

Highlights

In conclusion, peptides play an important role in the development of nanovectors as they maintain many of their individual functions also in transfection complexes or in hybrid sequences. Peptides are used to condense nucleic acids, serve as scaffolds for the complex, are important for cell recognition, and mediate endocytosis, endosomal escape, as well as intracellular targeting. The field of peptide-nucleic acid transfection complexes or of liposomal vectors in combination with peptides is in fast development, and much progress toward non-viral transfection has been made even though a number of hurdles still need to be overcome for efficient *in vivo* applications. One of the greatest challenges will be to transpose the results obtained in cell cultures to humans where issues such as stability in serum as well as efficient and selective transport to the target sites have to be solved. As *in vitro* and *in vivo* experiments not always correlate, a better understanding of the processes that are responsible for such differences is necessary.

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Burkhard Bechinger obtained his PhD in 1989 at the Biocenter Basel, Switzerland. His thesis dealt with investigations of electrostatic interactions within lipid bilayers using membrane biophysical approaches. During his postdoctoral stay at the University of Pennsylvania (1990–1993) he developed and applied new solid-state NMR methods for the investigation of membrane-associated polypeptides. In 1993 he had the opportunity to start his own small research group at the Max Planck Institute of molecular Physiology in Dortmund, Germany with projects that were continued when he was nominated 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.