ACCOUNTS
of chemical research

Polymers for siRNA Delivery: Inspired by Viruses to be Targeted, Dynamic, and Precise

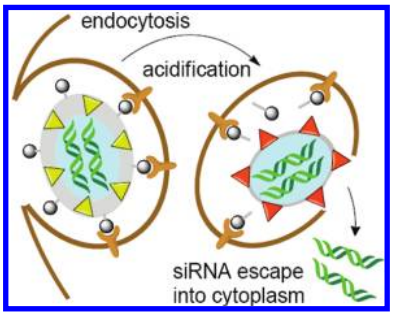
ERNST WAGNER*

Pharmaceutical Biotechnology, Center for System-Based Drug Research, and Center for Nanoscience (CeNS), Ludwig-Maximilians-Universität München, D-81377 Munich, Germany

RECEIVED ON AUGUST 29, 2011

CONSPECTUS

Synthetic small interfering RNA (siRNA) presents an exciting novel medical opportunity. Although researchers agree that siRNA could have a great therapeutic impact, the required extracellular and intracellular delivery of these molecules into the disease-associated target cells presents the primary roadblock for the broader translation of these molecules into medicines. Thus, the design of adequate delivery technologies has utmost importance. Viruses are natural masterpieces of nucleic acid delivery and present chemists and drug delivery experts with a template for the design of artificial carriers for synthetic nucleic acids such as siRNA. They have been developed into gene vectors and have provided convincing successes in gene therapy. Optimized by biological evolution, viruses are programmed to be dynamic and bioresponsive as they enter living cells, and they carry out their functions in a precisely defined sequence. However, because they are synthesized within living cells and with naturally available nucleotides and amino acids, the chemistry of viruses is limited. With the use of diverse synthetic molecules and macromolecules, chemists can provide delivery solutions beyond the scope of the natural evolution of viruses.



This Account describes the design and synthesis of "synthetic siRNA viruses." These structures contain elements that mimic the delivery functions of viral particles and surface domains that shield against undesired biological interactions and enable specific host cell receptor binding through the presentation of multiple targeting ligands. For example, cationic polymers can reversibly package one or more siRNA molecules into nanoparticle cores to protect them against a degradative bioenvironment. After internalization by receptor-mediated endocytosis into the acidifying endosomes of cells, synthetic siRNA can escape from these vesicles through the activation of membrane-disruption domains as viruses do and reach the cytoplasm, the location of RNA interference.

This multistep task presents an attractive challenge for chemists. Similar to the design of prodrugs, the functional domains of these systems have to be activated in a dynamic mode, triggered by conformational changes or bond cleavages in the relevant microenvironment such as the acidic endosome or disulfide-reducing cytoplasm. These chemical analogues of viral domains are often synthetically simpler and more easily accessible molecules than viral proteins. Their precise assembly into multifunctional macromolecular and supramolecular structures is facilitated by improved analytical techniques, precise orthogonal conjugation chemistries, and sequence-defined polymer syntheses. The chemical evolution of microdomains using chemical libraries and macromolecular and supramolecular evolution could provide key strategies for optimizing siRNA carriers to selected medical indications.

1. Introduction

Novel RNA therapeutics such as synthetic small interfering RNA (siRNA)[1,2] and microRNA,[3,4] longer double-stranded and conditional cytotoxic RNA,[5,6] or chemically modified mRNA[7] have created exciting medical opportunities. For translation into medical use, carriers for extracellular and intracellular delivery are required, as the free forms are only inefficiently taken up by target cells and rapidly cleared or degraded by the host. Viral vectors, where therapeutic nucleic acid replaces most of the virus genome, have dominated classical gene therapies for good reasons, they are far more potent than synthetic systems. Adenovirus associated viral vectors have recently proven to correct genetic forms of blindness in patients,[8] and genetic

Published on the Web 12/22/2011 www.pubs.acs.org/accounts
10.1021/ar2002232

modification of blood stem cells by retroviral vectors has rescued children affected by severe combined immunodeficiency.[9] Thus, viruses present natural masterpieces of nucleic acid delivery with recently proven clinical benefit for patients. Further genetic and chemical modifications of viral vectors[10,11] will provide potent molecular medicines.

The optimization of viruses however is also caught within the limits of their protein and lipid biosynthesis in living cells. Viral vectors are limited to certain sizes and type of their payload (natural nucleic acids); viral protein antigens and several viral nucleic acids are constitutively recognized by the host immune system. Thus, synthetic carriers,[12] if effective, may have distinct advantages over viral vectors and are the only delivery option for chemically modified medical nucleic acids. In the design of such synthetic carriers, we may learn from natural viruses.[13−15] Viruses are programmed to be dynamic and bioresponsive in their infection process. They activate different delivery functions at the different times when needed. During their biosynthesis, they have their nucleic acid packaged into nanosized cores, but the cores disassemble after cell entry. Viruses learned to survive in the host, they contain surface proteins which are recognized by host cell receptors. Alternatively, they may also capture host proteins at their surface which then mediate receptor-mediated uptake of the virus into endolysosomal vesicles of cells. From there, in a Trojan horse approach and triggered by special viral domains, the nucleic acid core is able to escape the rather hostile, degradative endolysosomal compartment and enter the cytoplasm of target cells.

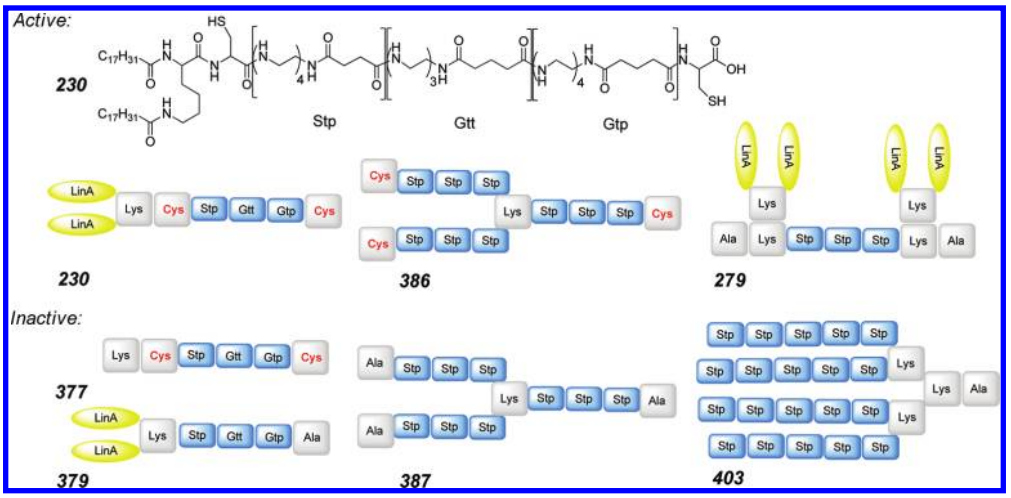
Chemists can design "synthetic viruses" containing domains which mimic viral functions.[11,13−17] Capitalizing on the diverse space of synthetic molecules, the chemical mimics of functional virus domains can be simpler and synthetically easier accessible than viral proteins, as already demonstrated in several examples.[17−19] Chemists may also learn from the sequence-defined precision of the assembly of functional virus domains into macromolecular and supramolecular structures. The million years biological evolution of viruses by gene shuffling and mutations cannot be directly translated into chemistry, but the individual functional delivery domains can be optimized using combinatorial chemistry and library screening.[20,21] Further on, macromolecular evolution can be persued by shuffling the obtained delivery domains into various defined precise sequences.[22,23] High throughput microreactors[24] can be used for screening supramolecular synthetic virus architectures.

2. Packaging Nucleic Acids Into Compact Nanoparticles

Reversible nucleic acid condensation by cationic proteins is a common natural process, for example, in packaging of whole mammalian genomes into chromatin, or RNA into organelles. Compaction is also a key function of viral cores for protection against the degradative environment during infection. Reversibility is important; the delivered nucleic acid has to be accessible for subsequent transcription. Polyionic interactions, hydrogen bonding, and hydrophobic interactions control the condensation of nucleic acids. In electrostatic complexes of plasmid DNA (pDNA) with polycations such as polylysine (pLys)[25] or polyethylenimine (PEI),[26] neutralization of approximately 10,000 negative phosphate charges of one pDNA molecule by approximately 100 polycation molecules[27] results in compaction into "polyplexes" with sizes of 20 to >100 nm (depending on aggregation events). While such compaction is important for delivery of large pDNA, it is not relevant for the much smaller siRNA (8 nm in length); small pLys or PEI polyplexes of 10−20 nm can be easily generated upon proper stabilization.[28,29] Electrostatic stabilization of siRNA polyplexes however is weaker than thatfor pDNA; with only 42−46 anionic charges, siRNA cannot present the stabilizing polyanionic string that pDNA provides with 10 000 charges. Addition of 0.5 M sodium chloride is sufficient for dissociation of siRNA polyplexes with 25 kDa branched PEI (BPEI), whereas the 2-fold salt concentration is required for dissolving pDNA polyplexes.[30] For pDNA transfer, 22 kDa linear PEI (LPEI) presents one of the most effective transfection agent. Despite a slightly lower polyplex stability, it is more effective than BPEI;[31,32] intracellular polyplex disassembly appeared as decisive factor. For siRNA, the low stability of standard siRNA polyplexes of LPEI makes siRNA transfer far less effective.[33]

Strategies for stabilization of siRNA polyplexes include (i) multimerization of siRNA into larger polyanions by RNA hybridization[34] or chemical ligation,[35] (ii) coformulation of siRNA with pDNA[36] or other polyanions, (iii) cross-linking of electrostatically bound polycations by bioreversible disulfide bonds[23,37] or covalent linkage of siRNA,[38,39] and (iv) hydrophobic stabilization.[23,40−43] Examples of hydrophobic stabilization include modification of BPEI with the amino acid tyrosine which provides an efficient siRNA transfection agent.[40] Modification of 20% of nitrogens reduces the solubility of BPEI, thereby stabilizing siRNA polyplexes. Analogously, modification of 800 Da oligoethylenimine with

SCHEME 1. siRNA Carriers Containing Stabilizing Hydrophobic Domains and Disulfide-Forming Cysteines[a 23]



[a]Top: Active siRNA carriers (i-shape *230*, three-arm *386*, U-shape *279*). Bottom: Ineffective siRNA carriers (*377*, *379* analogues of *230*; *387* analogue of *386*; four-arm *403*). LinA: linolic acid.

10 hexyl acrylates[41] or of triethylentetramine with 5 dodecyl acrylates[42] resulted in effective hydrophobically stabilized cores for siRNA delivery.

In our recent work, we compared defined triethylenetetramine (tt) and tetraethylenepentamine (tp) containing polycations for their efficacy in siRNA delivery.[23] Scheme 1 compares active polymer sequences (top) with analogous ineffective structures (bottom). Oligo(aminoamides) including three-armed (***387***) or four-armed (***403***) structures with up to 100 nitrogens cannot transfect siRNA. Modification with two terminal cysteines (which form disulfide linkages within polyplexes) and a hydrophobic domain, introduced for example as di(linolic acid)-modified lysine, results in polymers which efficiently package and deliver siRNA into cells, resulting in gene silencing (for example, polymer ***230***). Deletion of the hydrophobic domain (polymer ***377***) or the cysteines (***379***) results in loss of activity. Introduction of additional cysteines, for example, a third cysteine into three-armed structures (***386***), or introduction of a second hydrophobic domain (***279***) provides potent siRNA carriers.[23]

The polymer/siRNA core may be regarded as the engine of the delivery vehicle; for efficient and specific delivery, like in natural viruses, additional domains for cell entry and endosomal escape are required.

3. Targeting and Shielding: The Trojan Horse Approach

Viruses are optimized for surviving in the relevant body fluids. Their surface is decorated with ligands for attachment to their target cell surface receptors. Often they use more than one receptor type for intracellular uptake into host cells. Sometimes viruses are coated with blood factors which then act as endogenous targeting ligands for entry. Adenovirus normally uses CAR receptor as primary and integrins as secondary receptors; coating with coagulation factor X is responsible for a third pathway resulting in transfection of hepatocytes.[44]

Synthetic nanoparticles can utilize such multivalent recognition and cell uptake mechanisms for nucleic acid delivery.[45] For example, active targeting of siRNA to liver hepatocytes is possible with *N*-acetylgalactosamine ligands,[38,46] or endogenous LDL receptor-mediated targeting after association of lipid nanoparticles with plasma apolipoprotein E.[46] Targeting ligands can influence cell entry kinetics. The ligand epidermal growth factor (EGF), which actives macropinocytosis of cells, accelerates cellular uptake, resulting in 50% internalization of EGF/PEI/pDNA polyplexes within 5 min.[47] Synergistic dual targeting was observed with PEI/pDNA polyplexes containing two different peptidic ligands, a RGD peptide for integrin targeting and peptide B6 for transferrin receptor targeting. RGD dominated in cell surface binding, B6 in intracellular uptake.[48]

The chemist's Trojan horse approach provides opportunities not available for natural viruses. First of all, hydrophilic polymers such as polyethylene glycol (PEG) represent efficient agents for nanoparticle surface shielding against unintended interactions with the host.[18,49,50] Interactions with plasma complement and other proteins, or blood and immune cells can extinguish any benefit of "active targeting" ligands. Shielding is therefore indispensable for systemic

targeted delivery via the blood circulation. In cancer therapy, PEGylated polyplexes with elongated plasma circulation may take advantage of the "enhanced permeability and retention" (EPR) effect.[51] Long-term circulating nanoparticles can extravasate and passively accumulate at tumor sites due to the leakiness of tumor vessels and ineffective lymphatic efflux ("passive tumor targeting").

A second advantageous chemical option is the use of chemical ligands instead of immunogenic viral or other targeting proteins. The prostaglandin I2 analogus Iloprost, targeting the prostacyclin receptor IP1,[19] has successfully been applied for receptor-mediated transfection of lung cells. Other examples include the vitamin folic acid applied covalently bound for tumor-targeted siRNA delivery[52] or anisamide for targeting the tumor-associated sigma receptor.[53]

4. Intracellular Barriers: Escape from the Endosome

siRNA polyplex stability and endocytic uptake by target cells are important delivery tasks, the entrapment in the hostile endolysosomal vesicles presents the most critical hurdle, and degradation by lysosomal enzymes in an acidic environment is the dead end for a very significant fraction of delivered nucleic acid. Endosomal escape is particularly important for the delivery of nuclease-sensitive siRNAs which have to reach the cytoplasm of ideally all target cells in sufficient amounts. Viruses have acquired efficient solutions for escaping from the maturating acidifying endosomes. For example, glycoproteins of enveloped viruses such as influenza virus contain hidden fusion peptides which are exposed after endocytosis, to trigger fusion of the viral with the endosomal membrane. Endocytosed nonenveloped viruses such as rhinovirus or adenovirus expose lytic domains which directly disrupt the endosomal membrane, either (in case of rhinovirus) generating a pore large enough for crossing of the viral RNA strand into the cytoplasm or (in case of adenovirus) disrupting the whole endosome. Such lytic domains have been utilized in artificial settings as synthetic peptides for endosomal escape of polyplexes.[13,54] Also other nature-derived or artificial lytic peptides were applied.[29,55−58] Beyond peptides, amphipathic lytic polymers have been incorporated.[38,59,60] To avoid premature lysis of cell surface membranes which would kill the host cell, these synthetic agents should become lytic only after endosomal entry, for example, triggered by endosomal acidification (see section 5).

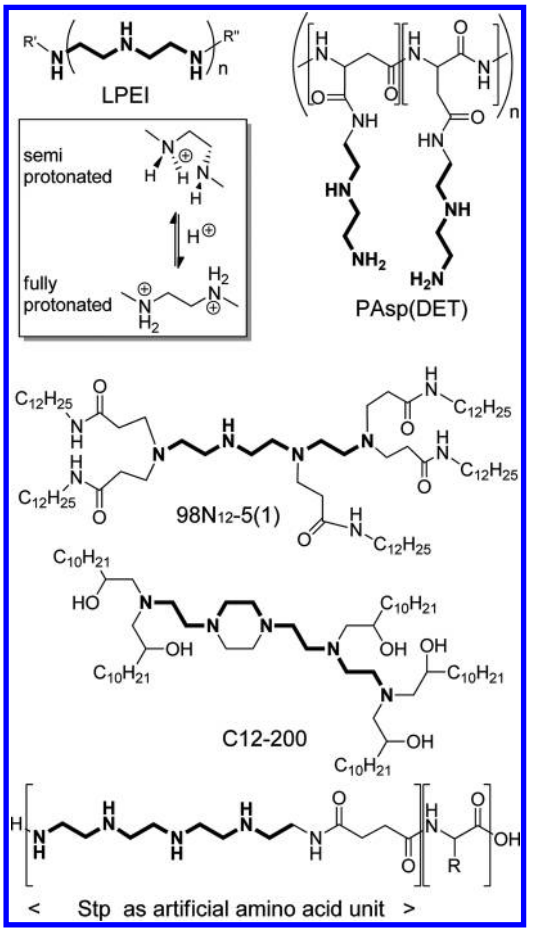
The antimalaria drug chloroquine, a weak lipophilic base which can cross cell membranes but becomes entrapped in the endolysosomes upon protonation, was first introduced by Cotten et al. to prevent lysosomal degradation of pDNA polyplexes.[61] Impressive effects were seen in K562 leukemia cells which due to a genetic defect have particularly strong acidification and consequently also very high chloroquine accumulation in endosomes, triggering osmotic vesicle swelling. Chloroquine was also found to synergize with membrane-active peptides. Recently, a cell-penetrating peptide was converted into an efficient siRNA carrier through covalent attachment of a chloroquine analogue.[57]

To capitalize on endosomal buffering and osmotic swelling, Behr and colleagues screened cationic polymers with "proton sponge" characteristics,[17] that is, buffer capacity between physiological neutral and endolysosomal pH. They discovered polyethylenimine (PEI) as very potent transfection polymer.[26] In contrast to polycations such as pLys which are already fully protonated at neutral pH, the proton sponge PEI displays incomplete protonation of nitrogens (about 50% for PEI associated with pDNA).[62] The increased density of positive charges upon endosomal acidification leads to an influx of chloride and water, resulting in osmotically triggered vesicle burst.[63] In contrast to unmodified pLys, derivatives such as histidinylated pLys or Lys/His copolymers also present proton sponges and efficient pDNA and siRNA carriers.[64,65]

Proton sponge activity however is not sufficient for endosomal escape; pDNA binding proton sponges are described which do not efficiently transfect.[66] Even for PEI polyplexes, surface shielding by PEGylation strongly reduces endosomal escape and transfection potency.[16,29] Apparently, in addition to endosomal buffering and osmotic pressurizing, the protonated positively charged polymer has to be exposed to directly interact with and destabilize the lipid membrane. Thus, efficient endosomal escape still presents a bottleneck and, especially for PEI, the window between effective endosomolytic and cytotoxic dose is narrow. In the case of BPEI, cytotoxicity can be reduced by modification of 10% nitrogens with succinic acids, thus converting nitrogens into negatively charged carboxylate groups. Suc-PEI is still a proton sponge, containing protonable carboxylates and nitrogens. Due to its biocompatibility, higher doses can be applied and efficient siRNA delivery obtained under conditions where BPEI is inactive.[30] Similarly, tyrosine-modified BPEI, which is less soluble at neutral pH but becomes soluble upon endosomal protonation, mediates potent siRNA transfer.[40]

PEI has a special position within organic polymers; the aminoethylene unit (if fully protonated) provides one of the highest possible positive charge densities. As the diaminoethane motif (Scheme 2) is only partly protonated at physiological pH but further protonated within endosomes, it has unique properties both as endosomal protone sponge and in membrane destabilization, responsible for high transfection activity of PEI derivatives.[26,31,67] Protonation of one diaminoethane nitrogen triggers electronic and steric effects on the neighboring nitrogens. The theoretical distribution of distances between nearest neighbor nitrogens of LPEI[62] shows a maximum of 0.29 nm for unprotonated and semiprotonated PEI (presenting entangled gauche conformations, with two neighboring nitrogens sharing one protonation), whereas fully protonated LPEI show exclusively stretched, antiperiplanar conformation of nitrogens presented by a peak maximum of 0.38 nm. Not surprisingly, the diaminoethane motif appears also in other potent transfection agents (Scheme 2).[42,43,68] For example, biodegradable polyapartamide (pAsp) amidated in the side chain carboxyl groups with diethylentriamine (DET) is a highly effective pDNA carrier, with far superior activity over analogous polyapartamide modified with diaminopropane (DPT) units.[68] Similarly, oligoethylenimine-modified polypropylenimine dendrimers (containing the diaminoethane motif) were far more potent in transfection than analogous polypropylenimine (i.e., diaminopropane units)-modified dendrimers.[67] The diaminoethane motif is also present in carriers $98N_{12}$-5(1),[42] and C12−200,[43] (see section 6).

SCHEME 2. Proton-Sponge Diaminoethane Motif in Efficient Nucleic Acid Carriers[a]



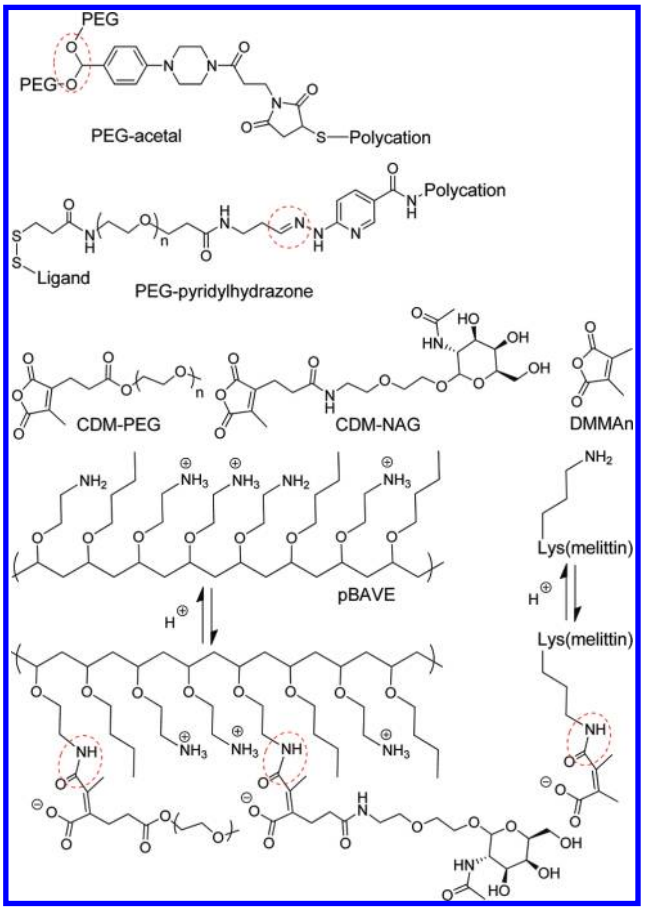
[a]PEI,[26] pAsp(DET),[68] $98N_{12}$-5(1),[42] C12−200,[43] Stp unit.[23,74]

5. Optimizing the Timing: Dynamic and Biodegradable Polymers

Like natural viruses, their synthetic analogues have to activate their different delivery functions just at the right time and location. As mentioned, nanoparticle shielding by PEGylation is beneficial in the blood circulation before attachment to the target cell, but can be a serious impediment for cell-surface binding and endosomal escape. Conversely, lytic activity may be required within endosomes, but can trigger severe toxicity if acting already at the cell surface. Polymer/siRNA cores should be stable in the extracellular environment, but should disassemble within the cytoplasm to release the siRNA in an accessible form required for gene silencing. Such location- and time-triggered sequence of activities can be programmed into nanoparticles[69] by utilizing chemistries which sense their microenvironment (for example, pH-labile bonds cleavable in the endosome, disulfide bonds reduced in the cytoplasm). Kinetics of such chemistries have to be compatible with the individual and the whole delivery process: for example, endosomal cleavage to occur within 30 min, but extracellular stability to last for hours.

Dynamic shielding with PEG and pH-triggered endosomal deshielding of polyplexes has been achieved by incorporating acetal linkages,[59,70] pyridylhydrazone,[16,71] or dialkylmaleic acid[38] bonds into polymer−PEG conjugates (see Scheme 3). Such a reversible PEGylation was crucial in receptor-targeted delivery of PEI polyplexes, for maintaining endosomal escape and efficient transfection which was 10−100-fold higher than with irreversibly PEGylated polyplexes.[16,71] A dialkylmaleic acid linker (CDM) was applied for reversibly PEG shielding and receptor targeting of the amphipathic poly(butyl/amino-vinylether) pBAVE.[38] Upon pH-triggered endosomal deshielding the amphipathic lytic polymer is unmasked, resulting in endosomolysis and delivery of covalently attached siRNA into the cytoplasm.

SCHEME 3. pH-Sensitive Linkages and Dynamic Conjugates[a] [16,29,38,39,70,71]



[a]Acid-labile linkages highlighted with dashed circles.

A disulfide linkage between siRNA and polymer was applied cleavable in the reducing cytoplasm. Another dynamic siRNA conjugate with dual-responsive characteristics was designed by covalent disulfide linkage of siRNA to a PEG-pLys conjugate.[39] As pLys does not significantly mediate endosomal escape, its conjugate with pH-reversibly masked melittin peptides (modified with dimethylmaleic acid anhydride, DMMAn) was applied. Exposure to endosomal pH recovers the lytic activity of melittin, resulting in effective cytoplasmic delivery and release of siRNA.

6. Chemical Evolution: Find the Needle in the Haystack

In searching for synthetic carriers which are most suitable for a specific medical application (with a defined tissue and disease indication), for obvious reasons chemists cannot rely on the natural and genetic evolution of viruses. Nevertheless, important underlying principles can be extracted: discovery of potent microdomains by chemical evolution (this section), combined with shuffling of these functional microdomains into macromolecular and supramolecular structures (next section).

Optimization of cationic lipid/pDNA complexes (lipoplexes) for therapy of cystic fibrosis presents an early example of chemical evolution which resulted in a synthetic formulation applied in clinical gene therapy studies.[72,73] Screening of the numerous cationic lipid formulations in a relevant system (instillation into the mouse lung in vivo) was important to discover the most effective lipid **#67**, a cholesterol derivative conjugated with spermine in a T-shape configuration, which was coformulated with a stabilizing PEG-lipid. Clinically less relevant in vitro screening using cultured cells did not provide the same results.

During the past decade Akinc, Anderson, Langer, Lynn, and colleagues[20,21,42,43] reported impressive achievements in chemical evolution of polymeric and lipopolymeric carriers. Semiautomated syntheses of thousands of polymers were combined with high-throughput transfection screening of pDNA or siRNA complexes. Applying robust Michael addition chemistry, multiple combinations of amines and hydrophobic (di)acrylates were evaluated, resulting in libraries of poly(β-aminoesters) for pDNA delivery and lipophilic modified oligoamines (lipidoids) for siRNA delivery. Alternatively, lipidoid libraries were generated by addition of oligoamines to lipophilic alkyl-epoxides. Interesting siRNA carriers were coformulated with stabilizing lipids and evaluated in vivo for gene silencing in mouse livers. Two potent structures, $98N_{12}$-5(1)[42] and C12-200[43] contain the diaminoethane motif (Scheme 2).

7. Molecular Evolution: Precise Macromolecular Sequences and Supramolecular Assemblies

Further molecular evolution into multifunctional supramolecular structures goes beyond classical combinatorial or parallel chemistry. However, like in "gene shuffling" of genetic evolution, identified functional delivery microdomains can be organized into various assembly sequences and structures, followed by screening and selection of the most effective assemblies. Such a molecular evolution only can work if the process occurs in defined, reproducible form.

For example, based on recent technology of solid-phase supported synthesis of sequence-defined polymers[22,74] and the knowledge of the high potency of the oligomeric diaminoethane motif (sections 2 and 4), our group has synthesized libraries of precise, sequence-defined pDNA and siRNA carriers (Scheme 1). These carriers include additional

functional domains, such as stabilizing disulfide-forming cysteines or hydrophobic modifications in defined sequence and topologies (T-shapes, i-shapes, U-shapes).[23] Extending the concept, also PEG modules and receptor targeting ligands can be incorporated for targeted delivery.

In addition to precise covalent assembly, novel technologies for supramolecular assembly are expected to greatly impact further optimization of synthetic virus architectures. These include layer-by-layer assemblies[75] and microfluidic assemblies.[24] Using a microreactor, a supramolecular library of 648 pDNA nanoparticles was generated extremely fast (within <3 h).

8. Conclusion and Perspective

Now is the golden age for designing synthetic viruses. The present knowledge on molecular structure and function of natural viruses and a more than two decades rising learning curve on synthetic carriers provides an excellent starting position. Importantly, modern chemistry with refined orthogonal conjugations and highly sensitive, high-resolution analytics in vitro and in vivo is able to tackle the challenging tasks of macromolecular and supramolecular assembly of synthetic viruses. Not to forget that they are strongly needed for further development of nucleic acid medicines. There, beyond chemistry, the early definition of the appropriate host target tissue and relevant pharmacological disease model presents a key necessity in the evolution of synthetic siRNA carrier for the intended medical use.

I thank my Ph.D. students for designing siRNA carriers and Olga Brück for skillful assistance. Our work was supported by Excellence cluster NIM, Roche Kulmbach, and the Biotech Cluster m4T12.

BIOGRAPHICAL INFORMATION

Ernst Wagner received a doctoral degree in Organic Chemistry from the Vienna Technical University. Since 2001 he is full professor of Pharmaceutical Biotechnology at LMU Munich, and since 2005 a member of the Munich Center for Nanoscience. He coordinates the Area "Biomedical Nanotechnologies" of the Excellence Cluster "Nanosystems Initiative Munich".

FOOTNOTES

*E-mail: ernst.wagner@cup.uni-muenchen.de.

REFERENCES

1 Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001**, *411*, 494–498.

2 Vaishnaw, A. K.; Gollob, J.; Gamba-Vitalo, C.; Hutabarat, R.; Sah, D.; Meyers, R.; de Fougerolles, T.; Maraganore, J. A status report on RNAi therapeutics. *Silence* **2010**, *1*, 4.

3 Kota, J.; Chivukula, R. R.; O'Donnell, K. A.; Wentzel, E. A.; Montgomery, C. L.; Hwang, H. W.; Chang, T. C.; Vivekanandan, P.; Torbenson, M.; Clark, K. R.; Mendell, J. R.; Mendell, J. T. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* **2009**, *137*, 1005–1017.

4 Ibrahim, A. F.; Weirauch, U.; Thomas, M.; Grunweller, A.; Hartmann, R. K.; Aigner, A. MicroRNA Replacement Therapy for miR-145 and miR-33a Is Efficacious in a Model of Colon Carcinoma. *Cancer Res.* **2011**, *71*, 5214–24.

5 Schaffert, D.; Kiss, M.; Rodl, W.; Shir, A.; Levitzki, A.; Ogris, M.; Wagner, E. Poly(I:C)-mediated tumor growth suppression in EGF-receptor overexpressing tumors using EGF-polyethylene glycol-linear polyethylenimine as carrier. *Pharm. Res.* **2011**, *28*, 731–741.

6 Venkataraman, S.; Dirks, R. M.; Ueda, C. T.; Pierce, N. A. Selective cell death mediated by small conditional RNAs. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 16777–16782.

7 Kormann, M. S.; Hasenpusch, G.; Aneja, M. K.; Nica, G.; Flemmer, A. W.; Herber-Jonat, S.; Huppmann, M.; Mays, L. E.; Illenyi, M.; Schams, A.; Griese, M.; Bittmann, I.; Handgretinger, R.; Hartl, D.; Rosenecker, J.; Rudolph, C. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat. Biotechnol.* **2011**, *29*, 154–157.

8 Ashtari, M.; Cyckowski, L. L.; Monroe, J. F.; Marshall, K. A.; Chung, D. C.; Auricchio, A.; Simonelli, F.; Leroy, B. P.; Maguire, A. M.; Shindler, K. S.; Bennett, J. The human visual cortex responds to gene therapy-mediated recovery of retinal function. *J. Clin. Invest.* **2011**, *121*, 2160–2168.

9 Cavazzana-Calvo, M.; Fischer, A. Gene therapy for severe combined immunodeficiency: are we there yet? *J. Clin. Invest* **2007**, *117*, 1456–1465.

10 Boeckle, S.; Wagner, E. Optimizing targeted gene delivery: chemical modification of viral vectors and synthesis of artificial virus vector systems. *AAPS J.* **2006**, *8*, E731–E742.

11 Wagner, E. Converging Paths of Viral and Non-viral Vector Engineering. *Mol. Ther.* **2008**, *16*, 1–2.

12 Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. Design and development of polymers for gene delivery. *Nat. Rev. Drug Discovery* **2005**, *4*, 581–593.

13 Wagner, E.; Plank, C.; Zatloukal, K.; Cotten, M.; Birnstiel, M. L. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U.S.A* **1992**, *89*, 7934–7938.

14 Zuber, G.; Dauty, E.; Nothisen, M.; Belguise, P.; Behr, J. P. Towards synthetic viruses. *Adv. Drug Delivery Rev.* **2001**, *52*, 245–253.

15 Wagner, E. Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? *Pharm. Res.* **2004**, *21*, 8–14.

16 Walker, G. F.; Fella, C.; Pelisek, J.; Fahrmeir, J.; Boeckle, S.; Ogris, M.; Wagner, E. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. *Mol. Ther.* **2005**, *11*, 418–425.

17 Behr, J. P. The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia* **1997**, *51*, 34–36.

18 Zalipsky, S.; Mullah, N.; Harding, J. A.; Gittelman, J.; Guo, L.; Defrees, S. A. Poly(Ethylene Glycol) Grafted Liposomes With Oligopeptide or Oligosaccharide Ligands Appended to the Termini Of the Polymer Chains. *Bioconjugate Chem.* **1997**, *8* (2), 111–118.

19 Geiger, J.; Aneja, M. K.; Hasenpusch, G.; Yuksekdag, G.; Kummerlowe, G.; Luy, B.; Romer, T.; Rothbauer, U.; Rudolph, C. Targeting of the prostacyclin specific IP1 receptor in lungs with molecular conjugates comprising prostaglandin I2 analogues. *Biomaterials* **2010**, *31*, 2903–2911.

20 Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R. Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library *J. Am. Chem. Soc.* **2001**, *123*, 8155–8156.

21 Anderson, D. G.; Lynn, D. M.; Langer, R. Semi-Automated Synthesis and Screening of a Large Library of Degradable Cationic Polymers for Gene Delivery. *Angew. Chem., Int. Ed.* **2003**, *42*, 3153–3158.

22 Hartmann, L.; Hafele, S.; Peschka-Suss, R.; Antonietti, M.; Borner, H. G. Tailor-Made Poly(amidoamine)s for Controlled Complexation and Condensation of DNA. *Chemistry* **2008**, *14*, 2025–2033.

23 Schaffert, D.; Troiber, C.; Salcher, E. E.; Frohlich, T.; Martin, I.; Badgujar, N.; Dohmen, C.; Edinger, D.; Klager, R.; Maiwald, G.; Farkasova, K.; Seeber, S.; Jahn-Hofmann, K.; Hadwiger, P.; Wagner, E. Solid-Phase Synthesis of Sequence-Defined T-, i-, and U-Shape Polymers for pDNA and siRNA Delivery. *Angew. Chem., Int. Ed.* **2011**, *50*, 8986–8989.

24 Wang, H.; Liu, K.; Chen, K. J.; Lu, Y.; Wang, S.; Lin, W. Y.; Guo, F.; Kamei, K.; Chen, Y. C.; Ohashi, M.; Wang, M.; Garcia, M. A.; Zhao, X. Z.; Shen, C. K.; Tseng, H. R. A rapid pathway toward a superb gene delivery system: programming structural and functional diversity into a supramolecular nanoparticle library. *ACS Nano* **2010**, *4*, 6235–6243.

25 Wagner, E.; Cotten, M.; Foisner, R.; Birnstiel, M. L. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4255–4259.

26 Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.

27 The exact number depends on polymer size and nitrogen/DNA phosphate (N/P) ratio in polyplex formation. For PEI 22 kDa (~500 nitrogens), polyplexes are mixed at N/P ratio of 5 or higher, representing 100 (or more) polymer molecules per 5 kbp pDNA.

Approximately 50 polymer molecules (N/P 2.5, empirical value) are incorporated into the polyplex.

28 DeRouchey, J.; Schmidt, C.; Walker, G. F.; Koch, C.; Plank, C.; Wagner, E.; Radler, J. O. Monomolecular assembly of siRNA and poly(ethylene glycol)-peptide copolymers. *Biomacromolecules* **2008**, *9*, 724–732.

29 Meyer, M.; Philipp, A.; Oskuee, R.; Schmidt, C.; Wagner, E. Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *J. Am. Chem. Soc.* **2008**, *130*, 3272–3273.

30 Zintchenko, A.; Philipp, A.; Dehshahri, A.; Wagner, E. Simple Modifications of Branched PEI Lead to Highly Efficient siRNA Carriers with Low Toxicity. *Bioconjugate Chem.* **2008**, *19*, 1448–1455.

31 Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **2001**, *3*, 362–372.

32 Itaka, K.; Harada, A.; Yamasaki, Y.; Nakamura, K.; Kawaguchi, H.; Kataoka, K. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. *J. Gene Med.* **2004**, *6*, 76–84.

33 Kwok, A.; Hart, S. L. Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. *Nanomedicine* **2011**, *7*, 210–219.

34 Bolcato-Bellemin, A. L.; Bonnet, M. E.; Creusat, G.; Erbacher, P.; Behr, J. P. Sticky overhangs enhance siRNA-mediated gene silencing. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16050–16055.

35 Mok, H.; Lee, S. H.; Park, J. W.; Park, T. G. Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing. *Nat. Mater.* **2010**, *9*, 272–278.

36 Chang Kang, H.; Bae, Y. H. Co-delivery of small interfering RNA and plasmid DNA using a polymeric vector incorporating endosomolytic oligomeric sulfonamide. *Biomaterials* **2011**, *32*, 4914–4924.

37 Matsumoto, S.; Christie, R. J.; Nishiyama, N.; Miyata, K.; Ishii, A.; Oba, M.; Koyama, H.; Yamasaki, Y.; Kataoka, K. Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules* **2009**, *10*, 119–127.

38 Rozema, D. B.; Lewis, D. L.; Wakefield, D. H.; Wong, S. C.; Klein, J. J.; Roesch, P. L.; Bertin, S. L.; Reppen, T. W.; Chu, Q.; Blokhin, A. V.; Hagstrom, J. E.; Wolff, J. A. Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12982–12987.

39 Meyer, M.; Dohmen, C.; Philipp, A.; Kiener, D.; Maiwald, G.; Scheu, C.; Ogris, M.; Wagner, E. Synthesis and Biological Evaluation of a Bioresponsive and Endosomolytic siRNA-Polymer Conjugate. *Mol. Pharmaceutics* **2009**, *6*, 752–762.

40 Creusat, G.; Rinaldi, A. S.; Weiss, E.; Elbaghdadi, R.; Remy, J. S.; Mulherkar, R.; Zuber, G. Proton sponge trick for pH-sensitive disassembly of polyethylenimine-based siRNA delivery systems. *Bioconjugate Chem.* **2010**, *21*, 994–1002.

41 Philipp, A.; Zhao, X.; Tarcha, P.; Wagner, E.; Zintchenko, A. Hydrophobically modified oligoethylenimines as highly efficient transfection agents for siRNA delivery. *Bioconjugate Chem.* **2009**, *20*, 2055–2061.

42 Akinc, A.; Zumbuehl, A.; Goldberg, M.; Leshchiner, E. S.; Busini, V.; Hossain, N.; Bacallado, S. A.; Nguyen, D. N.; Fuller, J.; Alvarez, R.; Borodovsky, A.; Borland, T.; Constien, R.; de Fougerolles, A.; Dorkin, J. R.; Narayanannair, J. K.; Jayaraman, M.; John, M.; Koteliansky, V.; Manoharan, M.; Nechev, L.; Qin, J.; Racie, T.; Raitcheva, D.; Rajeev, K. G.; Sah, D. W.; Soutschek, J.; Toudjarska, I.; Vornlocher, H. P.; Zimmermann, T. S.; Langer, R.; Anderson, D. G. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* **2008**, *26*, 561–569.

43 Love, K. T.; Mahon, K. P.; Levins, C. G.; Whitehead, K. A.; Querbes, W.; Dorkin, J. R.; Qin, J.; Cantley, W.; Qin, L. L.; Racie, T.; Frank-Kamenetsky, M.; Yip, K. N.; Alvarez, R.; Sah, D. W.; de Fougerolles, A.; Fitzgerald, K.; Koteliansky, V.; Akinc, A.; Langer, R.; Anderson, D. G. Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1864–1869.

44 Alba, R.; Bradshaw, A. C.; Coughlan, L.; Denby, L.; McDonald, R. A.; Waddington, S. N.; Buckley, S. M.; Greig, J. A.; Parker, A. L.; Miller, A. M.; Wang, H.; Lieber, A.; van Rooijen, N.; McVey, J. H.; Nicklin, S. A.; Baker, A. H. Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood* **2010**, *116*, 2656–2664.

45 Ogris, M.; Wagner, E. To be targeted: is the magic bullet concept a viable option for synthetic nucleic acid therapeutics? *Hum. Gene Ther.* **2011**, *22*, 799–807.

46 Akinc, A.; Querbes, W.; De, S.; Qin, J.; Frank-Kamenetsky, M.; Jayaprakash, K. N.; Jayaraman, M.; Rajeev, K. G.; Cantley, W. L.; Dorkin, J. R.; Butler, J. S.; Qin, L.; Racie, T.; Sprague, A.; Fava, E.; Zeigerer, A.; Hope, M. J.; Zerial, M.; Sah, D. W.; Fitzgerald, K.; Tracy, M. A.; Manoharan, M.; Koteliansky, V.; Fougerolles, A.; Maier, M. A. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol. Ther.* **2010**, *18*, 1357–1364.

47 de Bruin, K.; Ruthardt, N.; von Gersdorff, K.; Bausinger, R.; Wagner, E.; Ogris, M.; Brauchle, C. Cellular dynamics of EGF receptor-targeted synthetic viruses. *Mol. Ther.* **2007**, *15*, 1297–1305.

48 Nie, Y.; Schaffert, D.; Rodl, W.; Ogris, M.; Wagner, E.; Gunther, M. Dual-targeted polyplexes: One step towards a synthetic virus for cancer gene therapy. *J. Controlled Release* **2011**, *152*, 127–134.

49 Plank, C.; Mechtler, K.; Szoka, F. C., Jr.; Wagner, E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **1996**, *7*, 1437–1446.

50 Merkel, O. M.; Librizzi, D.; Pfestroff, A.; Schurrat, T.; Buyens, K.; Sanders, N. N.; De Smedt, S. C.; Behe, M.; Kissel, T. Stability of siRNA polyplexes from poly(ethylenimine) and poly(ethylenimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by Fluorescence Fluctuation Spectroscopy and Single Photon Emission Computed Tomography (SPECT) imaging. *J. Controlled Release* **2009**, *138*, 148–159.

51 Maeda, H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv. Enzyme Regul.* **2001**, *41*, 189–207.

52 Thomas, M.; Kularatne, S. A.; Qi, L.; Kleindl, P.; Leamon, C. P.; Hansen, M. J.; Low, P. S. Ligand-targeted delivery of small interfering RNAs to malignant cells and tissues. *Ann. N.Y. Acad. Sci.* **2009**, *1175*, 32–39.

53 Nakagawa, O.; Ming, X.; Huang, L.; Juliano, R. L. Targeted intracellular delivery of antisense oligonucleotides via conjugation with small-molecule ligands. *J. Am. Chem. Soc.* **2010**, *132*, 8848–8849.

54 Zauner, W.; Blaas, D.; Kuechler, E.; Wagner, E. Rhinovirus-mediated endosomal release of transfection complexes. *J. Virol.* **1995**, *69*, 1085–1092.

55 Chen, C. P.; Kim, J. S.; Steenblock, E.; Liu, D.; Rice, K. G. Gene transfer with poly-melittin peptides. *Bioconjugate Chem.* **2006**, *17*, 1057–1062.

56 Boeckle, S.; Fahrmeir, J.; Roedl, W.; Ogris, M.; Wagner, E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Controlled Release* **2006**, *112*, 240–248.

57 Andaloussi, S. E.; Lehto, T.; Mager, I.; Rosenthal-Aizman, K.; Oprea, I. I.; Simonson, O. E.; Sork, H.; Ezzat, K.; Copolovici, D. M.; Kurrikoff, K.; Viola, J. R.; Zaghloul, E. M.; Sillard, R.; Johansson, H. J.; Said Hassane, F.; Guterstam, P.; Suhorutsenko, J.; Moreno, P. M.; Oskolkov, N.; Halldin, J.; Tedebark, U.; Metspalu, A.; Lebleu, B.; Lehtio, J.; Smith, C. I.; Langel, U. Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res.* **2011**, *39*, 3972–3987.

58 Sasaki, K.; Kogure, K.; Chaki, S.; Nakamura, Y.; Moriguchi, R.; Hamada, H.; Danev, R.; Nagayama, K.; Futaki, S.; Harashima, H. An artificial virus-like nano carrier system: enhanced endosomal escape of nanoparticles via synergistic action of pH-sensitive fusogenic peptide derivatives. *Anal. Bioanal. Chem.* **2008**, *391*, 2717–2727.

59 Murthy, N.; Campbell, J.; Fausto, N.; Hoffman, A. S.; Stayton, P. S. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Controlled Release* **2003**, *89*, 365–374.

60 Wakefield, D. H.; Klein, J. J.; Wolff, J. A.; Rozema, D. B. Membrane activity and transfection ability of amphipathic polycations as a function of alkyl group size. *Bioconjugate Chem.* **2005**, *16*, 1204–1208.

61 Cotten, M.; Langle-Rouault, F.; Kirlappos, H.; Wagner, E.; Mechtler, K.; Zenke, M.; Beug, H.; Birnstiel, M. L. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4033–4037.

62 Ziebarth, J. D.; Wang, Y. Understanding the protonation behavior of linear polyethylenimine in solutions through Monte Carlo simulations. *Biomacromolecules* **2010**, *11*, 29–38.

63 Sonawane, N. D.; Szoka, F. C., Jr.; Verkman, A. S. Chloride Accumulation and Swelling in Endosomes Enhances DNA Transfer by Polyamine-DNA Polyplexes. *J. Biol. Chem.* **2003**, *278*, 44826–44831.

64 Midoux, P.; Monsigny, M. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chem.* **1999**, *10*, 406–411.

65 Leng, Q.; Scaria, P.; Zhu, J.; Ambulos, N.; Campbell, P.; Mixson, A. J. Highly branched HK peptides are effective carriers of siRNA. *J. Gene Med.* **2005**, *7*, 977–986.

66 Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans-Nieuwenbroek, N. M.; Crommelin, D. J.; Hennink, W. E. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. *Biomacromolecules* **2004**, *5*, 32–39.

67 Russ, V.; Gunther, M.; Halama, A.; Ogris, M.; Wagner, E. Oligoethylenimine-grafted polypropylenimine dendrimers as degradable and biocompatible synthetic vectors for gene delivery. *J. Controlled Release* **2008**, *132*, 131–140.

68 Miyata, K.; Oba, M.; Nakanishi, M.; Fukushima, S.; Yamasaki, Y.; Koyama, H.; Nishiyama, N.; Kataoka, K. Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity. *J. Am. Chem. Soc.* **2008**, *130*, 16287–16294.

69 Wagner, E. Programmed drug delivery: nanosystems for tumor targeting. *Expert Opin. Biol. Ther.* **2007**, *7*, 587–593.

70 Knorr, V.; Allmendinger, L.; Walker, G. F.; Paintner, F. F.; Wagner, E. An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes. *Bioconjugate Chem.* **2007**, *18*, 1218–1225.

71 Fella, C.; Walker, G. F.; Ogris, M.; Wagner, E. Amine-reactive pyridylhydrazone-based PEG reagents for pH-reversible PEI polyplex shielding. *Eur. J. Pharm. Sci.* **2008**, *34*, 309–320.

72 Lee, E. R.; Marshall, J.; Siegel, C. S.; Jiang, C.; Yew, N. S.; Nichols, M. R.; Nietupski, J. B.; Ziegler, R. J.; Lane, M. B.; Wang, K. X.; Wan, N. C.; Scheule, R. K.; Harris, D. J.; Smith, A. E.; Cheng, S. H. Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **1996**, *7*, 1701–1717.

73 Zabner, J.; Cheng, S. H.; Meeker, D.; Launspach, J.; Balfour, R.; Perricone, M. A.; Morris, J. E.; Marshall, J.; Fasbender, A.; Smith, A. E.; Welsh, M. J. Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo 90. *J. Clin. Invest.* **1997**, *100*, 1529–1537.

74 Schaffert, D.; Badgujar, N.; Wagner, E. Novel Fmoc-polyamino acids for solid-phase synthesis of defined polyamidoamines. *Org. Lett.* **2011**, *13*, 1586–1589.

75 Blacklock, J.; Vetter, A.; Lankenau, A.; Oupicky, D.; Mohwald, H. Tuning the mechanical properties of bioreducible multilayer films for improved cell adhesion and transfection activity. *Biomaterials* **2010**, *31*, 7167–7174.