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Cationic siRNAs Provide Carrier-Free Gene Silencing in Animal Cells

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Small interfering RNA (siRNA)-mediated gene silencing has become a drug development paradigm.[1] It has entered clinical trials unusually fast, through chemical[2] and medical experience built on previous nucleic acid based drug candidates. Meanwhile it is also rediscovering the fact that nucleic acids per se do not enter intact human cells.

Cationic vectors developed earlier for gene delivery are multifunctional: they bind their cargo tightly *via* electrostatic forces; excess cationic charges borne by the nanoparticles so formed coaggregate anionic heparan sulfate proteoglycans (HSPG) of the cell surface,[3] thus triggering endocytosis. Subsequently, membrane-disturbing[4] or endosome-swelling[5] properties borne by the vector release some of the engulfed material into the cytoplasm.

siRNAs are a 100-fold smaller than genes. The cargo-to-carrier electrostatic binding is weakened to the same extent, favoring unwanted extracellular exchange of the small nucleic acid for any larger polyanion.[6] However, carriers are easier to link chemically to oligos than to genes. Such molecular conjugates[7] are expected to display a more classical pharmacology and a better efficiency/inflammation balance than supramolecular particles, including polyethyleneglycol-coated lipoplexes.[8,9]

Several oligocation-conjugated siRNAs have been described recently,[10−13] but all still bear a global negative charge and hence are unable to enter cells according to the mechanism described above. We recently developed a stepwise automated synthesis of oligodeoxyribonucleotide-oligospermines that allows incorporation of any number of spermine residues at any position of an oligonucleotide. It makes use of a DMT-spermine phosphoramidite[14] in addition to the four nucleic bases synthons. The synthesis was adapted to ribonucleic acids, and spermine coupling yields were improved for the synthesis of the larger oligomers used here (see Figure 1 and Supporting Information).

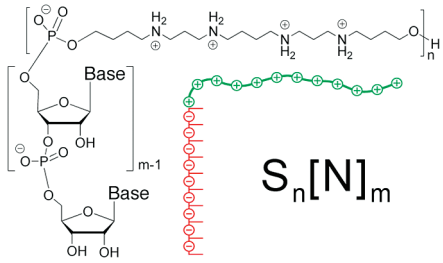

Figure 1. Structure of 5′-spermine-conjugated oligonucleotides $[N]_m$.

For proof-of-principle, we synthesized a fluorescent 3′-FAM 19-mer oligodeoxyribonucleotide 5′-conjugated to a stretch of 18 spermines $S_{18}[dN]_{19}$ (Figure 1) and observed its fate in HeLa cells under a fluorescence microscope (Figure 2a). After 4 h of incubation, cells showed, besides intracellular fluorescent vacuoles, a diffuse fluorescent halo throughout their cytoplasm, with a nuclear rim and nucleoli accumulation. Cell entry via HSPG-mediated endocytosis requires an overall cationic charge balance. Furthermore, subsequent endosome swelling and rupture require a fraction of nonprotonated amines as proton sponges.[15] Both conditions appeared fulfilled for $S_{18}[dN]_{19}$. To define the influence of the amine/phosphate balance on cell entry of oligonucleotide−oligospermine conjugates, we synthesized a series of fluorescent, nuclease-resistant, phosphorothioate oligonucleotides with an increasing number of 5′-appended spermine residues ($S_n[dN]_{12}$, n = 0, 2, 5, 8, and 11, Supporting Information). As expected, only long-term (24 h) incubation of HeLa cells with 5 μM $[dN]_{12}$ led to visible fluorescence accumulation (in lysosomes), presumably as a consequence of pinocytosis. $S_2[dN]_{12}$ showed intracellular distribution similar to $[dN]_{12}$ in these conditions (not shown).

A quite different, starry intracellular distribution was seen with $S_5[dN]_{12}$ even with shorter incubation times and lower concentra-

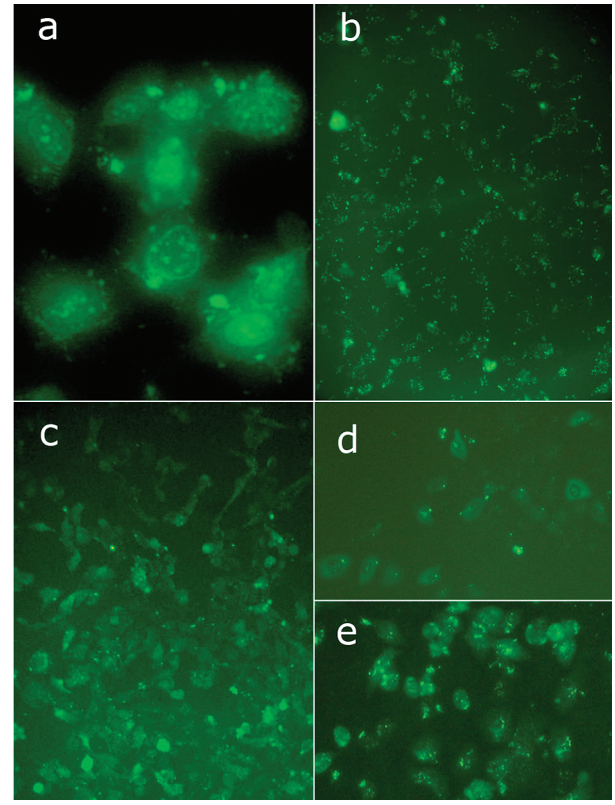

Figure 2. Intracellular distribution of fluorescent $S_n[dN]_m$ oligonucleotides. n, m, concentration (μM), incubation time (h); (a) 18, 19, 2, 4; (b) 5, 12, 2, 4; (c) 11, 12, 2, 0.5; (d) 11, 12, 2, 0.5; (e) 8, 12, 2, 4. (a−c) HeLa cells; (d, e) A549 cells.

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tions (Figure 2b). This distribution was reminiscent of endosome-trapped complexes observed using cationic vectors.[16] More interesting, $S_{11}[dN]_{12}$ led to robust and diffuse labeling of the cells (Figure 2c), reflecting endosome escape into the cytoplasm. This effect was confirmed on another cell type (A549 cells, Figure 2d). $S_8[dN]_{12}$ showed intermediate behavior, with intracellular patches distributed within a fluorescent cytoplasm (Figure 2e).

Carrier-mediated delivery is dependent on the carrier-to-nucleic acid charge ratio (N/P, since borne usually by amine and phosphate groups). Full amine protonation of $S_n[dN]_{12}$ is occurring as long as the ampholyte's total charge is not cationic.[17] $S_5[dN]_{12}$ (N/P = 1.25, see Figure 1) and upper homologues can therefore bind to polyanionic HSPGs and be taken up into endosomes. When the number of amines largely exceeds the number of phosphates, individual amine p*K*'s gradually decrease and become capable of acting as proton sponges. The diffuse cytoplasmic fluorescence pattern observed with $S_8[dN]_{12}$ (N/P = 1.6) indicates that oligonucleotide−oligospermine conjugates with the formal charge ratio N/P > 1.5 have indeed increased access to the cytoplasm.

siRNAs are 19-mer double-stranded RNA molecules with 3′-dT_2 overhangs that efficiently inhibit gene expression. Only the antisense strand is required during the target mRNA cleavage step. According to this and to the charge ratio required for cytoplasmic release, we conjugated 30 spermines 5′ to the siRNA sense strand (N/P = 1.7; Supporting Information). Endogeneous gene silencing was tested on A549Luc cells stably expressing large amounts of luciferase ((1−2) × 10^{10} RLU/mg protein). As shown in Figure 3,

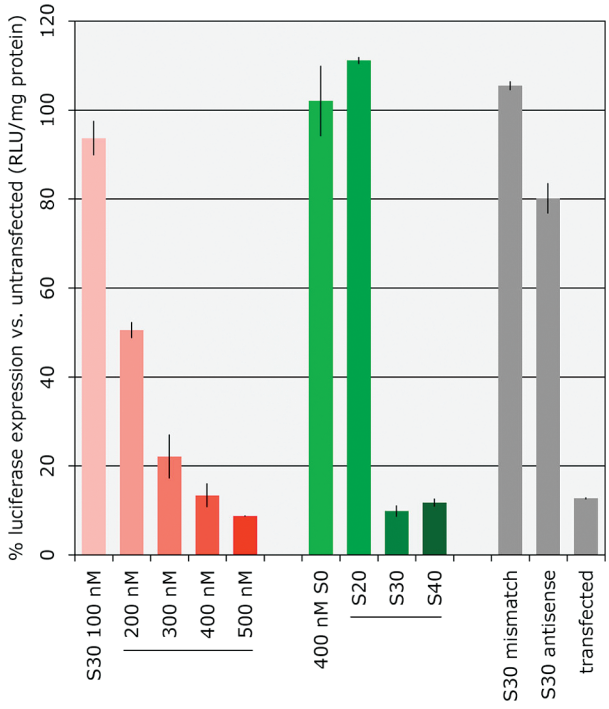

Figure 3. Luciferase gene silencing in A549Luc cells using 5′-sense oligospermine (Sn)-conjugated siRNA. Silencing is increasing with concentration (red bars) and number of spermine residues (green bars); gray bars: negative and positive controls.

a concentration-dependent luciferase expression knockdown was observed (S30, red bars), which was not observed with a shorter S20 tail and did not improve with a longer S40 tail (green bars). Negative control experiments using the "naked" S0 siRNA without spermine, or an S30 siRNA containing 3 sequence mismatches (gray bar), showed no luciferase silencing at 400 nM. Interestingly, conjugation of S30 5′ to the antisense strand resulted in loss of activity (Figure 3). This gives further credit to the silencing mechanism induced by Sn siRNA, since the antisense strand requires intracellular 5′-phosphorylation to become effective. Charge-dependent toxicity was observed in the absence of serum, as shown by a 33% (S30 siRNA) and 41% (S40 siRNA) decrease of cellular proteins 48 h after incubation with 400 nM oligonucleotide.

In summary, cationic spermine-conjugated siRNAs enter human cells and perform gene silencing in the submicromolar concentration range. Although less effective *in vitro* than recent cationic lipid formulations which sediment onto the cells (Figure 3, "transfected" gray bar using 10 or 100 nM siRNA), molecular siRNA drugs may outclass nanoparticles *in vivo*, where extracellular diffusion is a major concern. Whether cationic siRNAs will withstand *in vivo* conditions, particularly RNase degradation, rests on chemistry and further optimization. Work along these lines is in progress.

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Supporting Information Available: Synthesis of oligonucleotide−oligospermines and cell culture experiments; complete ref 9. This material is available free of charge via the Internet at http://pubs.acs.org.

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