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Synthesis and Nuclease Stability of Trilysyl Dendrimer–Oligodeoxyribonucleotide Hybrids

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Abstract—Hybrids of oligonucleotides and trilysyl-dendrimers with terminal acyl groups were prepared via solid-phase synthesis, including a DNA hexamer bearing an additional 3′-appendage. These were shown to be degraded more slowly by nuclease S1 than control strands, particularly at low pH, and, in one case, to form a duplex with a complementary strand whose melting point at pH 7 was higher than that of the control duplex.

Obtaining DNA in a form that renders it inaccessible to enzymatic degradation is one of the challenges in the emerging fields of gene therapy,[1] antisense[2] or antigene-therapy,[3] and chimeraplasty.[4,5] For short single-stranded DNA, modifications of the oligonucleotides themselves are commonly employed to make them more nuclease-resistant, rather than viral or non-viral carriers that entrap the nucleic acids. We have previously reported that cationic peptides[6] and porphyrins[7] provide a moderate protective effect on short unmodified oligonucleotides when engaging them in non-covalent complex formation. In order to increase the protective effect, two approaches have been evaluated: (i) covalently linking the potential ligands to the DNA strand to be protected, and (ii) appending more than one ligand in order to increase the total protective effect. Since branched cationic peptides[8] and polyamidoamine dendrimers[9] were known to aid the transfer of genetic material into cells, the ligands were appended to dendrimers. Here we show that quinolones, when covalently appended to dendrimer–oligonucleotide hybrids, can provide protection against nuclease attack for short oligonucleotides under acidic conditions, that is at a pH typically found in endosomes.[10] Short, modified oligonucleotides can have substantial biological activity.[11]

Tetramer **1** (Scheme 1) was the first target to be prepared. It contains four nucleotides and an appended trilysyl-dendrimer with four terminal amino groups for the attachment of the carboxylic acid residues envisioned to bind to the DNA. Derivatives **1a–d** were synthesized using the general protocol for 5′-modification reported previously.[12] Automated DNA assembly on controlled pore glass via the phosphoramidite protocol employing the 5′-*N*-monomethoxytrityl-protected 3′-phosphoramidite of 5′-amino-5′-deoxythymidine, after conventional deblocking, provided the free 5′-amine that was coupled with Fmoc-Lys(Fmoc)-OH under activation with HBTU, HOBT, and DIEA. The resulting aminoacylated oligomer was Fmoc-deprotected with piperidine in DMF, followed by a second coupling with Fmoc-Lys(Fmoc)-OH and a second deprotection. Peptide couplings with quinoline carboxylic acid, 4,8-dihydroxyquinoline carboxylic acid, or nalidixic acid gave the protected precursors of **1a–c**, from which the free oligonucleotide hybrids were liberated by treatment with NH_4OH at room temperature.[13] Similarly, biotin-bearing **1d** was prepared.

To evaluate whether the dendrimer appendages provide a protective effect, each of the hybrids **1a–c** was separately mixed with one equivalent of control tetramer **2** and each mixture (21 μM strand concentration) treated with nuclease S1 (EC 3.1.30.1, 0.125 u/μL) in ammonium sulfate buffer at 37 °C. The rate of degradation of the oligonucleotides was monitored by measuring the disappearance of the full length oligomer via quantitative MALDI-TOF mass spectrometry, as previously reported.[14] The three aromatic acid residues affected the survival of the dendrimer–DNA hybrids to a different extent (Fig. 1a–c). Whereas chinoline-bearing **1a** was attacked as

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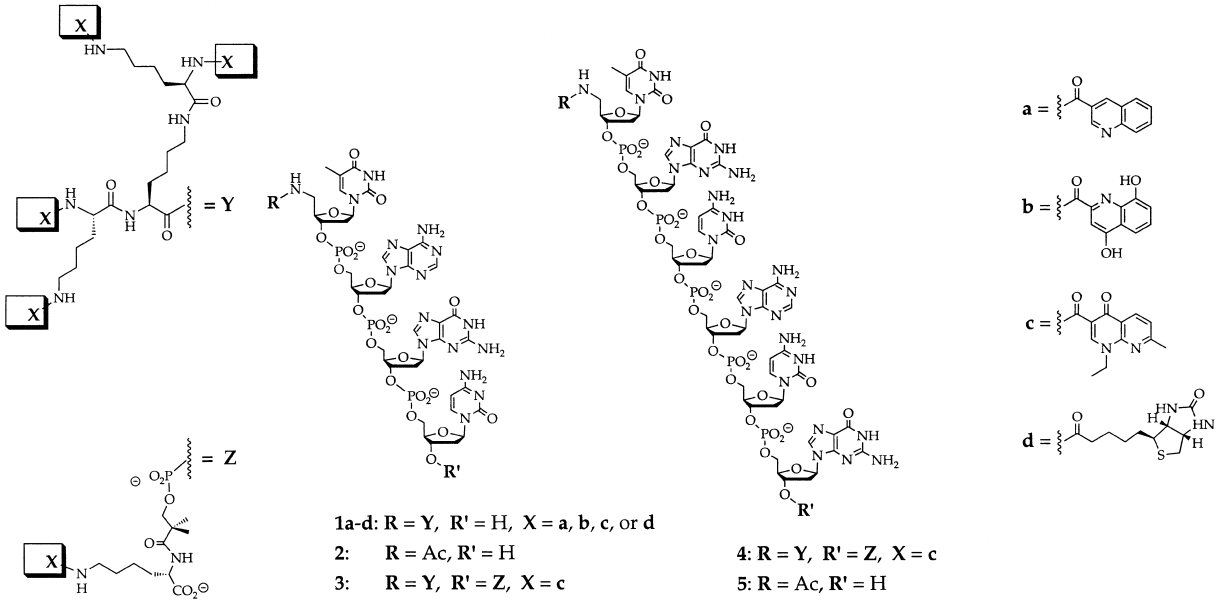

Scheme 1.

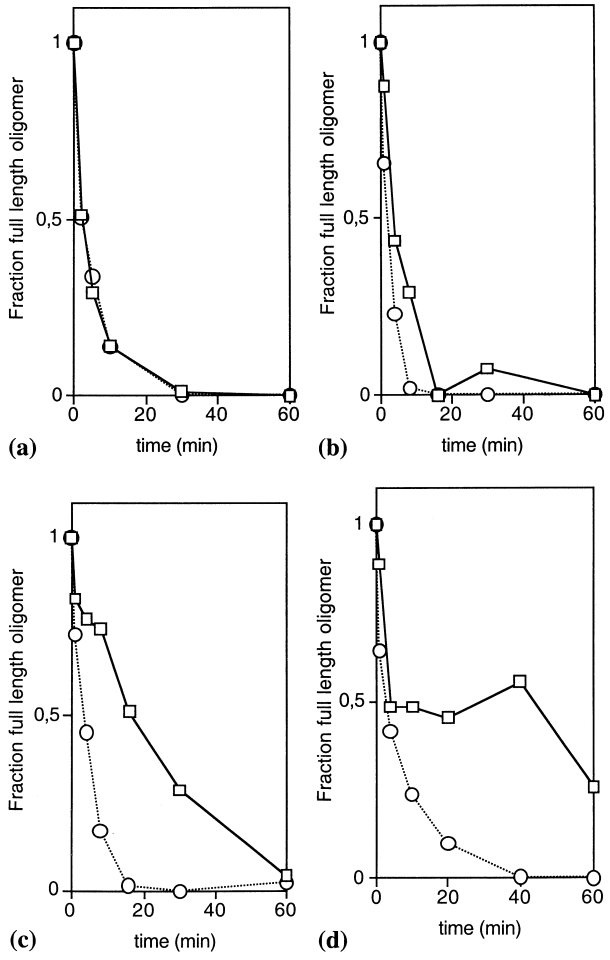

Figure 1. Kinetics of degradation of dendrimer–DNA hybrids by nuclease S1. Disappearance of full length oligonucleotides in an equimolar mixture of the hybrid (squares) and control tetramer **2** (circles) was monitored via MALDI-TOF mass spectrometry. Relative peak intensities to internal standard 5′-CGCATTAGCA-3′ from four spectra were averaged and normalized; typical standard deviation for data points: ±15%. (a) **1a** and **2**, (b) **1b** and **2**, (c) **1c** and **2**, and (d) **1d**, 2 equivalents of avidin, and **2**.

rapidly as **2**, the hybrid with dihydroxychinoline groups (**1b**) was slightly more stable than **2**, and **1c** with its nalidixic acid residues showed a more than 4-fold increase in half-life time. For comparison, treating a solution of **1d**, **2** (26 μM each) and avidin from hen egg white (47 μM) with nuclease S1 (7.5 u/μL) also resulted in some degradation, even though avidin, a tetrameric protein of 68 kDa, binds to biotin with a dissociation constant of 10^{-15} M and should thus generate an efficient steric shield for the DNA tetramer (Fig. 1d). During the first 5 minutes, the degradation of **1d** in the presence of avidin was faster than that of **1c**, whereas its long time survival was improved over that of **1c**.

Next, doubly modified 3′,5′-hybrid **3** (Scheme 1) was prepared in order to protect the still vulnerable 3′-terminus from exonuclease attack. Following a route established for 3′,5′-dipeptidyloligonucleotides,[15] Fmoc-Lys(Alloc)-OH was coupled to a triester support, Alloc-deprotected with Pd(0), and coupled with nalidixic acid. Fmoc-deprotection, coupling to 2,2-dimethyl-3-trityloxypropionic acid, and detritylation were followed by DNA synthesis, elaboration of the 5′-dendrimer portion, and release of **3**.[13] As expected, 3′-modified **3** was refractory to degradation by snake venom phosphodiesterase (EC 3.1.4.1). When an equimolar mixture of **1c**, **2**, and **3** was treated with nuclease S1 to compare their resistance to endonuclease attack, **3** was similarly resistant to endonuclease attack as **1c** (data not shown). In fact, **1c** was more long-lived in this mixture than without **3**, with >20% full length oligomer surviving after 60 min at 0.5 u/μL enzyme activity, even though **2** had almost completely disappeared after 10 min. This suggested that **1c** and **3** were interacting to form more nuclease-stable species. This effect was found to be pH-dependent, with a greater fraction of **1c** surviving 120 minutes nuclease attack at pH 5.4 than at pH 7.0.

This encouraged us to extended our work to an oligonucleotide long enough to bind to complementary

strands at physiological salt concentration. Hexanucleotide **4** (Scheme 1) was synthesized analogously to **3** on triester-derivatized cpg.[13,15] Besides being refractory to exonuclease attack, it showed an interesting resistance pattern when treated with nuclease S1. At near-neutral pH, the half-life of **4** under endonuclease attack was only about twice as long as that of control hexamer **5**. At pH 4.5, however, a substantial fraction of **4** appeared fully resistant to nuclease S1 and survived even 24 h of exposure to the endonuclease (Fig. 2). When acidification of a neutral solution of **4** was monitored via dynamic light scattering, the rapid formation of larger particles (>100 nm) was observed, followed by loss of signal. At high concentrations, a visible precipitate formed, leaving no detectable particles with diameters between 3 and 10^4 nm in solution. The precipitation was too rapid to determine the average particle size with the instrument available, which requires >3 minutes per measurement. This suggested that the resistance to nuclease attack observed at low pH was due to the formation of particles that deny the nuclease access to the DNA backbone. At neutral pH, however, **4** was found to bind to complementary DNA strand 5′-CGTGCA-3′ (**6**) with high affinity and without signs of aggregation. The UV–melting curve (Fig. 3) shows good cooperativity and strong hyperchromicity, indicating that the quinolone-terminated dendrimer portion does undergo a transition simultaneously with the DNA portion. The melting point of **4**:**6** at 40 μM strand concentration and 150 mM salt was 46 ± 1 °C, i.e. 10 °C higher than that of control duplex **5**:**6** that melted at 36 ± 1 °C. Further, one- and two-dimensional NMR-spectra of duplex **4**:**6** show the sharp signals expected for nucleotides in Watson–Crick duplexes, indicating that largely unaggregated species exist in neutral solution (supporting information). Further, NMR spectra of a solution of **4** and a target strand with a base mismatch did not show signs of duplex formation, indicating that the dendrimer appendage induces a high degree of base pairing fidelity.

Since all three quinoline derivatives tested in **1a**–**c** are, in principle, capable of intercalation, but only the dendrimers bearing nalidixic acid show the acid-mediated protective effect, it is likely that protonation plays an important role in the formation of particles. The pK_a of the protonated form of the imino group in free nalidixic acid is 6.0,[16] so that at lower pH, net-neutral, zwitterionic species will exist that are more likely to condense than oligoanionic strands that repel each other electrostatically. A pH of approximately 5.0 is found in endosomes that form in cells when large molecules are taken up via endocytosis.[10] Formation of particles that physically block the attack of nucleases is what many non-viral preparations induce in double-stranded DNA to be used for transfection,[17] as the particles not only prevent attack, but also facilitate uptake. The protective effects seen with **4** suggest that with dendrimers as appended ligands even very short single-stranded DNA, i.e. molecular species that are otherwise very difficult to formulate due to their low propensity to precipitate, can achieve increased nuclease resistance. In fact, they may undergo transitions between a protected form when in the acidic environment typically found in endosomes[10] and a monomeric form capable of binding complementary DNA or RNA when in a pH-neutral environment, such as the cytosol. Other forms of covalently linking ligands and oligonucleotides may also favor the formation of complexes via interactions that are otherwise too weak or unspecific to be used for tuning the bioavailability and recognition properties of oligonucleotides.

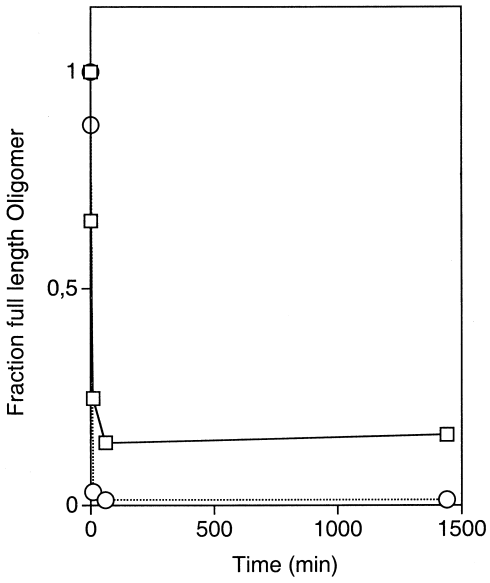

Figure 2. Kinetics of degradation of DNA-hybrid **4** (squares) and control strand **5** (circles) by nuclease S1 (1.4 u/μL) at pH 4.5, 2.6 mM strand concentration and 37 °C. Quantitation was via MALDI-TOF MS, using 5′-ACGTCAGTTAGCT-3′ as internal standard in the matrix preparation.

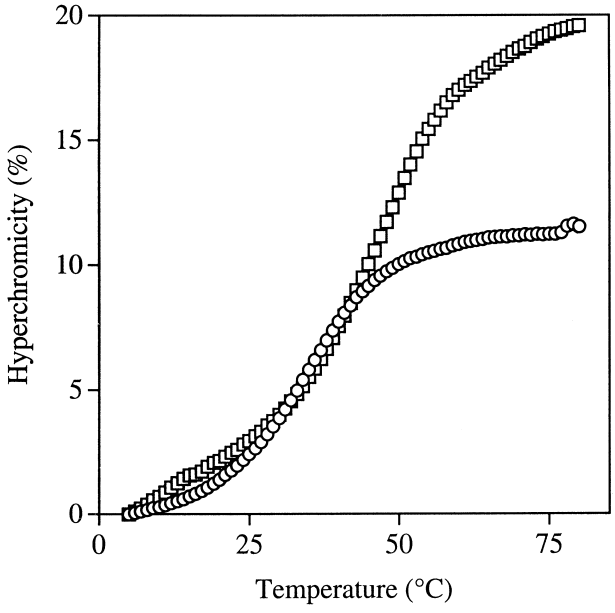

Figure 3. UV–melting curves of the duplexes **4**:**6** (squares) and **5**:**6** (circles) at 260 nm, 40 μM strand concentration, 150 mM NH_4OAc-buffer, pH 7, and a heating rate of 1 °C/min.

Supporting Information

The calibration plot for quantitative MALDI-TOF detection of **4**, one- and two-dimensional NMR spectra of the duplex **4**:**6**, and MALDI spectra of compounds **4**, **3**, and **2c** are available as supporting material.

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