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COMMUNICATIONS

Published on Web 12/13/2005

Triplex Formation with α-L-LNA (α-L-Ribo-Configured Locked Nucleic Acid)

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The specific recognition of homopurine−homopyrimidine tracts in duplex DNA by triplex-forming oligonucleotides (TFOs) provides an attractive strategy for genetic manipulation and is therefore of interest in medicinal chemistry and biotechnology.[1−4] In a triplex, the TFO is located in the major groove of a DNA duplex where it interacts with the purines in the duplex through Hoogsteen hydrogen bonding. In the parallel TFO binding motif, T:A•T and C:G•C$^+$ base triplets are formed.[5] However, formation of C:G•C$^+$ base triplets is pH dependent and disfavored at physiological pH, and thus, targeting of mixed G and A sequences becomes unfeasible at physiological pH. Despite considerable efforts over the past decade, the instability of pyrimidine motif triplexes at physiological pH continues to limit the biological applications.

For the development of ideal antisense and antigene molecules, a huge number of nucleic acid analogues have been studied. Recently, it was shown that locked nucleic acid (LNA)[6] (Figure 1) oligonucleotides have the ability to form triplexes at neutral pH.[7,8] The optimum thermostability was obtained with TFOs composed of alternating DNA and LNA monomers, while, intriguingly, fully modified LNA TFOs did not form triplexes at pH values above ∼6.[8] α-L-LNA is the α-L-ribo-configured stereoisomer of LNA (Figure 1). In duplex contexts, α-L-LNA-modified oligonucleotides stabilize hybridization with complementary DNA and RNA by up to ∼4 °C per modification.[9] With NMR studies, we have shown that α-L-LNA acts as a B-type (DNA) mimic when incorporated in Watson−Crick duplexes, as opposed to LNA, which acts as an A-type (RNA) mimic.[10,11]

In this study, we use UV melting experiments, electrophoretic mobility shift assays (EMSA), and CD and NMR spectroscopy to evaluate the triplex-forming capabilities of α-L-LNA oligonucleotides, in which the placement of modifications is systematically varied [α-L-LNA-modified oligonucleotides are available from Exiqon A/S (www.exiqon.com)].

In the CD spectra obtained at 10 °C and pH 6.8, we observe a negative band at ∼215 nm for each of the TFOs investigated (Figure 2 and Figure S1). [Experimental details are included in the Supporting Information.] This band confirms the formation of triplex for all TFOs.[12] The CD curves for the modified oligonucleotides generally appear similar to that of the unmodified triplex (**ON0**) except for the fully α-L-LNA-modified TFO (**ON9**).

EMSA conducted at pH 6.8 confirms the triplex-forming ability of the α-L-LNA-modified TFOs. Interestingly, the electrophoretic mobilities of the modified TFOs are slightly lower than that of the unmodified TFO (Figure 3). The CD spectra, though, show that no large-scale structural changes are occurring (except perhaps for the **ON9** triplex), and changes are probably restricted to local changes owing to the unnatural sugar−phosphate backbone of the α-L-LNA nucleotides.

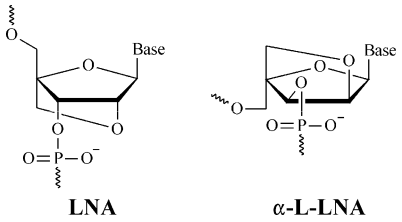

Figure 1. The chemical structure of LNA and α-L-LNA.

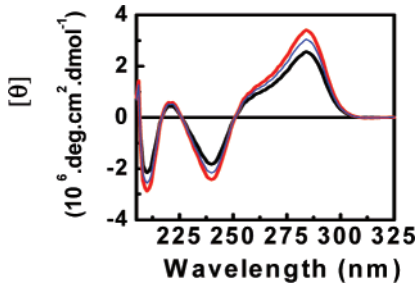

Figure 2. CD spectra of triplexes formed by **ON0** (black curve), **ON4** (red curve), and **ON5** (blue curve). Triplex concentration in CD experiments was 8 μM.

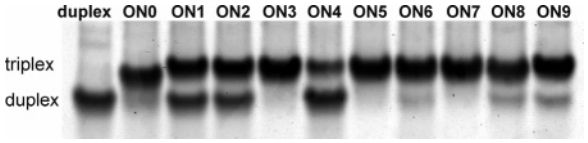

Figure 3. Electrophoretic mobility shift assay of triplex formation at pH 6.8.

Next, we determined the triplex thermal stabilities for the nine α-L-LNA TFOs (**ON1**−**ON9**) together with that for the unmodified reference TFOs (**ON0** and **ON0^{Me}**) at pH 6.8 (Table 1). The UV melting experiments showed biphasic curves which indicate a two-step melting process (Figure 4 and Figure S2). The duplex melting transition at 68.5 °C is identical within experimental uncertainty for all melting curves. The triplex melting temperatures vary within the series of modified TFOs but is in all cases increased significantly compared to the DNA reference. The largest absolute increase in thermostability is 24 °C (**ON4**), while the largest relative increase is 5.7 °C per modification (**ON8**). For synthetic reasons, the modified α-L-LNA cytidines are methylated at the 5-position in the nucleobase. We therefore compare melting temperatures with **ON0^{Me}**, in which all cytidines, except the 3′-terminal one, are methylated at the 5-position. However, methyl groups at the 5-position do not alter the structure of the triplexes (Figure S3), and hence we use **ON0** as reference in other experiments.

From our data, it is not possible to identify clear-cut design rules for α-L-LNA TFOs as there appears to be some sequence dependence. However, as general rules, one should modify every third or fourth nucleotide in the TFO and optimize the number of

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10.1021/ja055483r CCC: $33.50

Table 1. Thermal Stability of α-L-LNA-Modified Triplexes at pH 6.8 (α-L-LNA Nucleotides Are Shown in Bold Red)[a]

		T_m	ΔT_m	ΔT_m/mod
ON0	5'-CTC TTC TTT TCT TTC	27.0		
ON0^{Me}	5'-C̲T̲C TT**C̲** TTT T**C̲**T TTC	33.5		
ON1	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	55.3	21.8	3.1
ON2	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	45.5	12.0	1.7
ON3	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	47.0	13.5	2.7
ON4	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	57.4	23.9	4.8
ON5	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	53.2	19.7	4.9
ON6	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	47.3	13.8	3.5
ON7	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	51.8	18.3	4.6
ON8	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	50.6	17.1	5.7
ON9	5'-**C**T**C** T**T**C **T**T**T** T**C**T **T**T**C**	41.5	8.0	0.6

[a] Melting temperatures, T_m; changes in melting temperatures relative to **ON0^{Me}**, ΔT_m; and changes in melting temperatures per α-L-LNA modification, ΔT_m/mod; all in °C. **C̲** is 5-methylcytidine. The 15-mer TFOs were targeted to a 23 base pair dsDNA duplex.

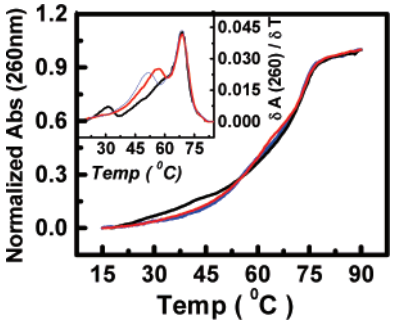

Figure 4. The UV melting profiles of the triplexes formed with **ON0^{Me}** (black curve), **ON4** (red curve), and **ON5** (blue curve). The inset shows the corresponding first derivative curves. Triplex concentration in melting experiments was 1 μM.

C α-L-LNA modifications to draw on the extra stabilization of the 5-methyl group.

In the UV curves, we observe some hysteresis in the region of the triplex transitions, indicating that the kinetics of the TFO association process is too slow to reach equilibrium at the heating and cooling rate we use (0.5 °C/min). Analysis of the nonequilibrium UV melting and annealing curves allowed us to determine the association and dissociation constants for the duplex/triplex equilibrium using a two-state model and, subsequently, the thermodynamic parameters of TFO association (Table S1).[13] Complete thermodynamic profiles at 15 °C show that the favorable free energy of α-L-LNA triplex formation is the result of a favorable enthalpy term exceeding an unfavorable entropy contribution. The larger exothermic formation enthalpies for the modified triplexes suggest that the hydrogen bonding and/or stacking interactions could be different from those in DNA triplexes. Kinetic analysis revealed that the increase in thermodynamic stability of the α-L-LNA triplexes is due to decreased dissociation rates of the modified TFO.

Finally, we concluded our studies by acquiring 1D ^{1}H NMR spectra of the triplexes formed by **ON0**, **ON4**, and **ON5** (Figure S4). The minute amounts of α-L-LNA at hand precluded any detailed analysis using 2D NMR experiments. Nonetheless, some information can be extracted from the imino regions of the spectra obtained at 15 and 25 °C. Three signals in the 14.5−16 ppm region are observed for the **ON4** and **ON5** triplexes. Chemical shifts in this spectral region are indicative of protonation of N3 of TFO cytidines. Presumably, the three nonterminal cytidines are protonated. For the unmodified triplex, these signals are only observed at 15 °C and are very broadened by exchange. The appearance of signals from H3 protons at 25 °C is a testimony of the increased thermostability of the α-L-LNA-modified triplexes.

Overall, sharp imino signals are seen for the modified triplexes. This shows that well-defined triplexes are formed. All imino signals resonate in the expected regions for Watson−Crick and Hoogsteen base pairing and the spectra of the triplexes possess similarities with the imino region of the DNA duplex on its own. Thus, it is likely that normal Watson−Crick and Hoogsteen base pairs are formed in the α-L-LNA triplexes.

In this study, we have presented the first evidence of triplex formation with α-L-LNA-modified TFOs. Furthermore, the α-L-LNA-modified triplexes are stable at physiological pH. In therapeutic applications, a TFO would be competing with specific DNA binding proteins for duplex access. The gain in triplex stability obtained by incorporation of the α-L-LNA modifications could prove to be essential when the objective is to inhibit transcription at the initiation or elongation stage by preventing either formation or progression of the transcription complex. Our results offer some hints as to the design of optimum α-L-LNA-modified TFOs and also hints that this design could be somewhat different than that of optimum LNA TFOs.[14]

Acknowledgment. We thank The Danish National Research Foundation for financial support. S.M. and N.K. acknowledge CSIR for funding. N.K. was supported by a Marie Curie Early Stage Research Training Fellowship of the European Community's Sixth Framework Program under Contract Number MEST-CT-2004-504018.

Supporting Information Available: Experimental details along with CD spectra, nondenaturing gel electrophoresis, UV melting profiles of all triplexes, and 1D ^{1}H NMR spectra of the triplexes formed with **ON0**, **ON4**, and **ON5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA055483R