Locked Nucleic Acid Technology™:

A brief overview

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LNA™ Structure

Nucleic acid duplexes fall into two major conformational types, the A-type and the B-type, dictated by the puckering of the single nucleotides, a C3´-endo (N-type) conformation in the A-type and a C2´-endo (S-type) conformation in the B-type (Figure 1) (Saenger, 1984). The A-type is adopted by RNA when dsRNA duplex regions are found, whereas the dsDNA in the genome adopt a B-type. Targeting DNA and RNA with high binding affinity can be effectuated by a conformational restriction in an N-type conformation.

In LNA, the O2´ and the C4´ atoms are linked by a methylene group hereby introducing a conformational lock of the molecule into a near perfect N-type conformation. We now define LNA as oligonucleotides containing one or more of the 2´-O,4´-C-methylene-β-D-ribofuranosyl nucleosides called LNA monomers (Figure 1).

Figure 1

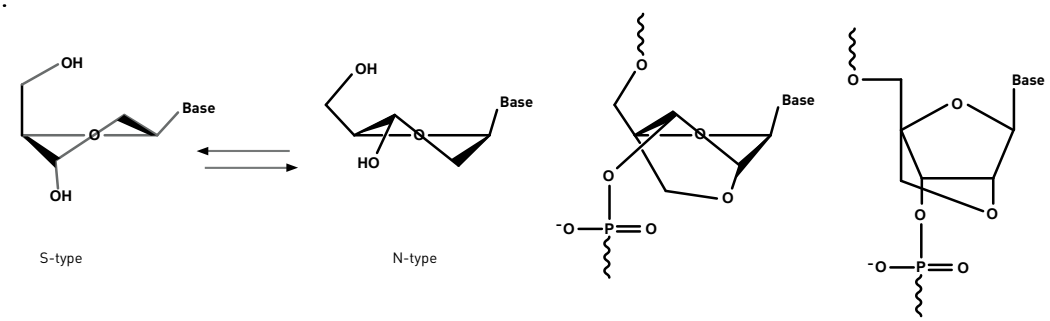

Figure 1. Nucleoside conformations and the structure and locked conformation of LNA monomers.

A major structural characteristic of LNA is its close resemblance to the natural nucleic acids. This leads to easy handling as LNA sequences have similar physical properties, including water solubility. Furthermore, LNA sequences are synthesised by the conventional phosphoramidite chemistry allowing automated synthesis of fully modified LNA-sequences as well as chimeras with DNA, RNA, modified monomers or labels.

A number of LNA:RNA and LNA:DNA hybrids have been structurally characterized by NMR spectroscopy and X-ray crystallography. In general, the duplexes retain the features common for native nucleic acid duplexes, i.e. usual Watson–Crick base pairing, nucleobases in the anti orientation, base stacking and a right-handed helical conformation. LNA monomers conformationally tune the flanking monomers, especially the 3'-flanking monomer, to adopt an N-type furanose conformation (Petersen and Wengel, 2003).

LNA Hybridization

The most conclusive feature of LNA is the unprecedented hybridisation to complementary nucleic acids. In the following, some general observations on LNA hybridization are listed (Petersen and Wengel, 2003).

- The increase in duplex stability by introducing one or several LNA monomers is strong in all cases with complementary RNA and in most cases with complementary DNA.
- The affinity-enhancing effect pr. LNA monomer is generally more pronounced against complementary RNA than against complementary DNA (normally from +3 to +7 °C against RNA and from +2 to +5 °C against DNA).
- LNA monomers incorporated in the 3´- or 5´-termini induce a smaller increase in thermal stability than do centrally placed LNA monomers.
- The relative increase in thermal stability per LNA monomer is largest for a single or a few non-consecutive incorporations in DNA/RNA/2'-OMe-RNA-sequences (mixmers).
- The relative increase in thermal stability per LNA monomer is larger in shorter sequences than in longer sequences.
- LNA monomers with pyrimidine bases tend to induce larger thermal stability increases than LNA monomers with purine bases (McTigue *et al.*,. 2004).
- LNA monomers introduced into phosphorthioate sequences also strongly stabilise duplexes.
- LNA monomers strongly stabilise triplexes and are useful in the design of triplex forming oligonucleotides (TFOs) composed of LNA mixmers with DNA.
- LNA: LNA duplexes are inherently very stable, and to avoid application-limiting self-hybridizing this should be carefully considered when designing LNA-sequences.

McTigue *et al.* have used a large set of LNA:DNA duplexes to suggest some predictive rules for hybridization enthalpy and entropy for all 32 possible nearest neighbours in LNA-DNA:DNA hybridization (McTigue *et al.*,. 2004). The thermodynamic data suggest that LNA can stabilize the duplex by either preorganization or improved stacking in terms of $\Delta\Delta S°$ or $\Delta\Delta H°$, but not both simultaneously. Other studies have similarly suggested a contribution to stability from either enthalpy or entropy (Obika *et al.*, 1998; Koshkin *et al.*, 1998; Christensen *et al.*, 2001)

LNA as RNA Targeting and Gene Silencing Molecule

LNA oligonucleotides have been investigated in a large number of biological studies. Many of these have been focused on gene silencing experiments following different RNA-targeting approaches such as steric block antisense, RNase H-mediated antisense, LNAzymes and siLNA. Additionally have cellular delivery, physiological stability as well as toxicity been studied (listed (Petersen and Wengel, 2003; Jepsen *et al.*, 2004; Vester *et al.*, 2004).

The issue of physiological stability mostly concerns the potential degradation of oligonucleotides by nucleases. A fully modified LNA sequence has been reported to be fully resistant towards the 3´-exonuclease SVPDE (Frieden *et al.*, 2003) whereas only minor protection against the same enzyme is obtained with one LNA monomer in the 3´-end or in the middle of a sequence. End-blocked sequences, i.e. LNA-DNA-LNA gapmers display a high stability in human serum compared to similar 2´-OMe modified sequences (Kurreck *et al.*, 2002). Another study showed that two terminal LNA monomers provided a significant protection against a Bal-31 exonucleolytic degration (Crinelli *et al.*, 2002). LNA oligonucleotides can be delivered into cells using standard cationic transfection

agents (Petersen and Wengel, 2003; Wahlstedt *et al*., 2000), and the usefulness of LNA as antisense oligomers has been amply demonstrated and reviewed (Petersen and Wengel, 2003; Jepsen *et al*., 2004; Vester *et al*., 2004).

In recent years siRNA has drawn much attention as a novel means of mediating gene silencing. LNA-modified siRNA constructs have been shown to be potent. A systematic study on LNA modified siRNAs thus showed that LNA 3´-end modifications substantially enhance serum half-life when evaluated relative to the unmodified siRNAs (Elmén *et al*. 2005), and that LNA-modified sense strands reduce sequence-related off-target effects. LNA modifications have furthermore proven essential for gene silencing by the so-called sisiRNA (short internally segmented interfering RNA) concept (Bramsen *et al*., 2007), which involves a nicked sense strand (and thus three RNA strands). In short, these results underline the promise of LNA-modified siRNA in relation to realizing the promises of RNAi for therapeutic applications (Elmén *et al*. 2005; Bramsen *et al*., 2007; Braasch *et al*. 2003).

LNA Probes

The properties of LNA for targeting both RNA and DNA make LNA an unprecedented tool for nucleic acid probes. Absolutely crucial in this context is the freedom in design with excellent co-operativity between LNA monomers and other classes of monomers and labels (Obika *et al*. 1998; www.exiqon.com). LNA-based probes have been used intensively for a wide range of purposes, and reviews have been published (Petersen and Wengel, 1998; Mouritzen *et al*. 2003). LNA has for example been applied as capture probe for direct isolation of RNA-sequences containing polyA-tracts (from cell extracts). A 20-mer polyT containing seven LNA nucleotides thus was successfully used to detect polyA-RNA accumulation within the nucleus/nucleolus of wild-type cells (Thomsen *et al*., 2005).

LNA-modified DNA probes have very convincingly been used for *in situ* detection of micro-RNAs in the zebrafish embryo (Wienholds *et al*. 2005; Kloosterman *et al*. 2006), and previously unidentified expression patterns of miRNAs in mouse embryos have been detected (Kloosterman *et al*. 2006). Optimal RNA-detection was found at hybridization temperatures 20-25°C below the melting temperature of the probe, and it was possible to use short probes (~14-mers) due to the high binding affinities induced by LNA monomers. Efficient LNA-probes at the 8-mer length have also been developed (Gamper *et al*. 2005).

LNA-based molecular beacons have proven advantageous for use because of high thermostability, enhanced selectivity and nuclease resistance. LNA-based molecular beacons can thus be used to detect SNPs and to increase the sensitivity of PCR (Sidon *et al*. 2006).

Summary

LNA has in a short time found widespread use in the development of therapeutics and for optimization of probes. Noteworthy, three LNA gapmers are in clinical trails for cancer indications (Santaris Pharma A/S and Enzon Pharmaceuticals, Inc.). The central feature of LNA is its high-affinity recognition of complementary nucleic acids. This enables the design of relatively short LNA sequences with unique nucleic acid recognition power.

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