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Current Topics

LNA (Locked Nucleic Acid): High-Affinity Targeting of Complementary RNA and DNA†

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ABSTRACT: Locked nucleic acid (LNA) is a nucleic acid analogue containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA mimicking sugar conformation. LNA oligonucleotides display unprecedented hybridization affinity toward complementary single-stranded RNA and complementary single- or double-stranded DNA. Structural studies have shown that LNA oligonucleotides induce A-type (RNA-like) duplex conformations. The wide applicability of LNA oligonucleotides for gene silencing and their use for research and diagnostic purposes are documented in a number of recent reports, some of which are described herein.

The first sections of this account describe some basic properties of LNA[1] (locked nucleic acid), whereas the latter sections focus on some of the recently described applications of LNA oligonucleotides. The intention of this contribution is to highlight properties and applications of LNA, while the reader is referred to recent reviews of LNA (*1*, *2*) for more comprehensive coverage.

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[1] Abbreviations: LNA, locked nucleic acid; T_m, melting temperature; PS, phosphorothioate; SVPD, snake venom phosphodiesterase; EGR-1, early growth response 1; ICAM-1, intercellular adhesion molecule 1; siRNA, small interfering RNA; TAR, HIV-1 transactivation responsive element; TFOs, triplex-forming oligonucleotides; SNPs, single-nucleotide polymorphisms; apoB, apolipoprotein B; MGB, minor groove binder; FISH, fluorescence *in situ* hybridization.

Hybridization Properties and Structural Features of LNA

We have defined LNAs (LNA oligonucleotides) as oligonucleotides containing at least one LNA monomer, i.e., one 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide (see Figure 1) (*3*, *4*). The LNA monomers adopt *N*-type sugar puckers, also termed the C3′-*endo* conformation (*3*, *5*). A number of structural analogues and configurational isomers of LNA have been synthesized over the past several years, but the focus herein is on LNA (β-D-*ribo* configuration) (*3*, *4*, *6*) and α-L-LNA (α-D-*ribo* configuration, a stereoisomer of LNA) (*7*) that have the following characteristics in common: (1) very efficient binding to complementary nucleic acids, (2) high potency as antisense molecules *in vitro* and *in vivo*, and (3) commercial availability as oligonucleotides (fully modified, mix-mers with DNA, RNA, other monomers, and various modifications) and as phosphoramidite building blocks. LNA oligonucleotides can be synthesized using conventional automated phosphoramidite chemistry, and LNA monomers are compatible with other monomers, e.g.,

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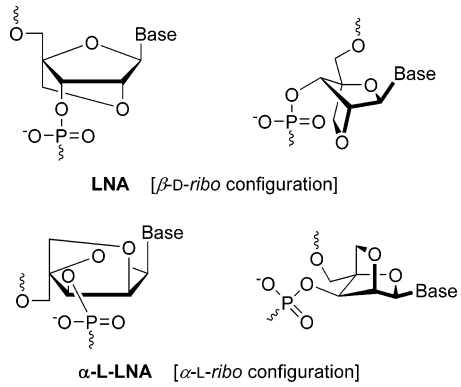


FIGURE 1: Structures of LNA and α-L-LNA monomers.

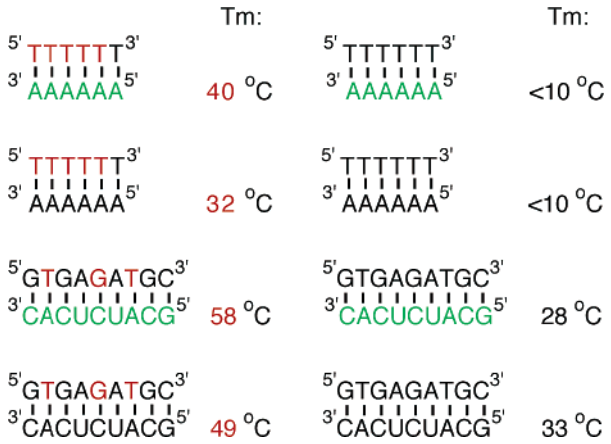


FIGURE 2: Some examples of melting temperatures (T_m values) for hybridization of LNA and DNA oligonucleotides to complementary RNA and DNA sequences (*4*). LNA monomers are red, DNA monomers black, and RNA monomers green.

DNA, RNA, and 2′-*O*-Me-RNA, and with phosphorothioate (PS) and/or phosphodiester linkages (*8*, *9*).

LNA resembles natural nucleic acids with respect to Watson−Crick base pairing. LNAs as fully modified oligomers or as mix-mers containing, for example, LNA and DNA or LNA and RNA nucleotides induce very high thermal stability of duplexes toward complementary RNA or DNA (*8*). This is illustrated with melting temperatures of some sequences in Figure 2. In addition, LNA·LNA base pairing, i.e., the self-annealing capacity, is so strong that it must be taken into account when designing LNAs (see www.exiqon.com for the LNA design tool). The thermal stabilities obtained for LNA oligonucleotides depend on the length of the sequence and the number of LNA nucleotides. Generally, the largest impact upon introduction of LNA nucleotides is observed for short oligonucleotides with one or more centrally positioned LNA monomers (*1*, *8*, *10*). Furthermore, single or multiple, but separated, LNA modifications appear to have a larger impact, in relative terms, than contiguous stretches of LNA nucleotides.

LNA·RNA and LNA·DNA hybrids have, in particular, been characterized by NMR spectroscopy. The duplexes display the features common for native nucleic acid duplexes, i.e., Watson−Crick base pairing, nucleobases in the *anti* orientation, base stacking, and a right-handed helical conformation. Three 9-mer LNA/DNA·RNA hybrids, in which the LNA/DNA mix-mer strand contained one, three, and nine LNA nucleotides have been studied (*11*, *12*). The study

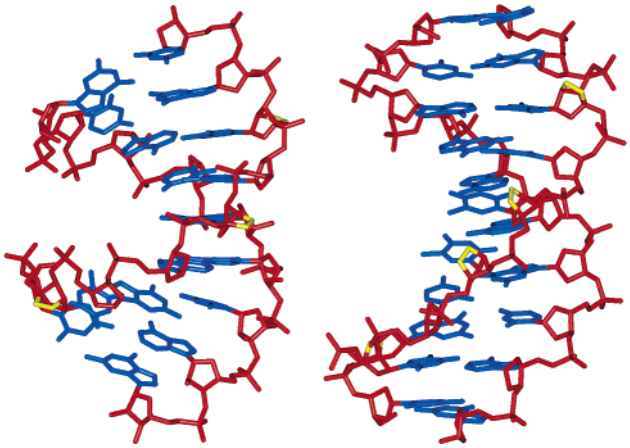


FIGURE 3: High-resolution NMR structures of LNA·RNA and α-L-LNA·DNA duplexes. The LNA(LNA/DNA mix-mer)·RNA duplex at left adops an A-type conformation (*11*), whereas the α-L-LNA(α-L-LNA/DNA mix-mer)·DNA duplex at right adopts a B-type conformation (*14*).

shows an increasing A-like character in the hybrids with an increase in the LNA content of the LNA strand. The fully modified LNA·RNA hybrid adopts A-type geometry (*12*) as observed for RNA·RNA hybrids. Remarkably, the hybrid with three modifications was shown to adopt a near-canonical A-type duplex geometry, indicating that the LNA nucleotides conformationally tune the DNA nucleotides in the LNA strand to attain *N*-type sugar puckers (*11*), as illustrated in Figure 3. A similar but localized effect was observed in the LNA/DNA·RNA hybrid with one modification, as the LNA nucleotide was shown to perturb the sugar puckers of the neighboring DNA nucleotides, predominantly in the 3′-direction of the LNA nucleotide (*11*).

When LNA modifications are incorporated into dsDNA duplexes, a general trend similar to that described above is observed, albeit with some alterations. Generally, the B-like character of the duplexes decreases upon incorporation of LNA modifications. Studies have been performed with 9- and 10-mer LNA/DNA·DNA hybrids, in which the LNA/DNA mix-mer strand contained one, three, or four LNA nucleotides (*13*). The sugar conformations of the nucleotides flanking an LNA monomer showed significant contributions from the *N*-type conformation. It was proposed that the increased stability of an LNA·DNA duplex relative to a DNA·DNA duplex is caused by a favorable entropy contribution due to the preorganized LNA combined with more efficient stacking of the nucleobases. Together, these structural studies designate LNA to be an RNA mimic also with regard to helical structure.

Similar studies have been performed with 9- and 10-mer α-L-LNA/DNA·DNA duplexes, containing three or four α-L-LNA nucleotides (*14*), and they retain the common features of native nucleic acid duplexes mentioned above. In general, the α-L-LNA/DNA·DNA duplexes adopt *S*-type sugar puckers and thus B-type helical structure (see Figure 3). The α-L-LNA·DNA 9-mer with three modifications has also been structurally studied in a duplex with complementary RNA (*15*). The geometry of the hybrid is intermediate between A- and B-type, and the global structure is very similar to that of an unmodified hybrid. Thus, α-L-LNA can be designated a DNA mimic with regard to helical structure.

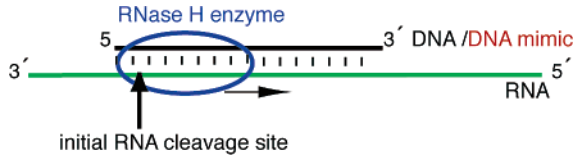


FIGURE 4: Illustration of the model of nuclease RNase H digestion of RNA·DNA hybrids. The vertical arrow indicates that the enzyme will cleave the RNA strand further toward its 5′-end until the enzyme senses the end of the hybrid.

Susceptibility of LNA Oligonucleotides to Nucleases

LNA oligonucleotides have a high potential for diagnostic and therapeutic uses. This implies contact with various media containing nucleases, for example, in serum samples or directly in cells. In general, it is advantageous that modified oligonucleotides are unnatural to such an extent that nucleases do not recognize them as substrates. One exception is RNase H-mediated cleavage of antisense oligonucleotides. The RNA strand of DNA·RNA duplexes is cleaved by RNase H, and it is often considered advantageous if an antisense oligonucleotide is compatible with RNase H cleavage (illustrated in Figure 4).

The difference in minor groove width of dsDNA, DNA·RNA, and dsRNA duplexes is believed to be the key recognition element for RNase H binding and RNA strand cleavage (*16*). The fact that very few fully modified oligonucleotides support RNase H-mediated cleavage has promoted investigation of so-called gap-mer oligonucleotides composed of modified segments flanking a central DNA (or phosphorothioate DNA) segment and other mixed constructs. Studies of LNAs and RNase H activity have reported somewhat contradictory results. Wahlestedt *et al*. (*17*) found that both an LNA/DNA/LNA gap-mer, with a six-nucleotide DNA gap, and an LNA/DNA mix-mer, with six DNA and nine LNA nucleotides interspersed, elicited RNase H activity, albeit for the mix-mer at a very slow rate. In another study (*7*), no RNase H-mediated cleavage was observed with a fully modified 11-mer LNA or with an 11-mer LNA/DNA mix-mer. Kurreck *et al*. (*18*) investigated various LNA/DNA mix- and gap-mers and found that a gap of six DNA nucleotides is necessary for 65% RNase H activity relative to the isosequential native DNA, and that a gap of seven DNA nucleotides allows complete RNase H activity. Frieden *et al*. (*19*) concluded from their study, which also included α-L-LNA, that an optimal DNA gap size is between 7 and 10 nucleotides for LNA/DNA/LNA gap-mers. Our own unpublished results show that a DNA stretch of five nucleotides flanked by LNA mixed sequences can support RNase H cleavage, albeit weakly, and that efficient cleavage is obtained with seven DNA nucleotides in the gap. RNase H cleavage depends on the sequence, length, and modification pattern of the oligonucleotide, the buffer, the temperature, and the activity of the enzyme which complicates comparison of different studies. The overall conclusion is that antisense LNA oligonucleotides can be designed to elicit RNase H activity while still containing LNA monomers for improved binding and target accessibility (*2*).

The situation is different for other nucleases for which resistance toward cleavage is actually advantageous. Complete stability against the 3′-exonuclease snake venom phosphodiesterase (SVPD) is reported for a fully modified LNA (*20*), while a significant increase in 3′-exonucleolytic stability was observed when the 3′-end was blocked with two LNA monomers (LNA/DNA mix-mers). On the contrary, no or only a very minor protection is induced with one penultimate LNA nucleotide or with a single LNA monomer in the middle of a sequence (*21*, *22*). Dissimilarly, one penultimately 3′-end-positioned α-L-LNA nucleotide provides a significant protection against SVPD that was further improved for an oligonucleotide containing two α-L-LNA nucleotides (α-L-LNA/DNA mix-mers) (*19*).

S1 endonuclease susceptibility was also investigated, and fully modified LNA and fully modified α-L-LNA are very stable against S1 endonuclease; however, when a DNA gap is present this will be cleaved (*19*). The serum stability of LNA oligonucleotides was tested, and LNA mix-mers were found to be very stable and LNA/DNA/LNA gap-mers to be much more stable than DNA alone (*17*). A similar conclusion was reported by Kurreck *et al*. (*18*), who found that oligonucleotides with LNA in the ends are more stable in human serum than the corresponding oligonucleotides composed of phosphorothioate DNA gaps and 2′-*O*-methyl flanks. DNAse I endonuclease degradation of end-modified 30-mer dsDNA showed that incorporation of one or two terminal LNA nucleotides was sufficient for a marked increase in stability (*23*). Bal-31 exonucleolytic degradation of the same oligonucleotides showed that one terminal LNA nucleotide did not provide protection whereas two terminal LNA nucleotides provided significant protection (*23*).

Polymerases usually display some nuclease activity, and their effects on LNA oligonucleotides have been observed (*24*) while investigating extension from primers with a few LNA monomers near or at the 3′-end. The presence of a single 3′-terminal LNA nucleotide significantly slowed degradation by the 3′−5′ proofreading exonuclease of DNA polymerases Pfu and Vent, whereas the exonuclease function of T7 DNA polymerase and KF was not significantly impeded. When a single LNA nucleotide is moved to the penultimate position, however, complete resistance to degradation is observed for all the enzymes that were tested, including exo III (*24*).

LNA Targeting RNA

One manner of sequence specific single cleavage of RNA is in hand. DNAzymes are catalytically active DNA molecules, some of which can cleave RNA site specifically after binding to complementary sequences. The 10−23 motif DNAzyme (Figure 5) was found by *in vitro* selection (*25*). It consists of a 15-nucleotide catalytic core flanked by two binding arms that control the specificity of the DNAzyme by hybridization to the RNA substrate. The cleavage requires a pyrimidine·purine sequence at the cleavage site. Incorporation of two LNA nucleotides in each of the binding arms of such a DNAzyme yielded an LNAzyme with a highly enhanced efficiency of RNA cleavage (*26*). The cleavage of highly structured targets (a 58-nucleotide RNA with known secondary structure and a 23S ribosomal RNA at 2904 nucleotides) was especially highly improved, but an enhancement was also seen with a 17-nucleotide minimal substrate. These LNAzymes showed multiple turnovers both with the 17-nucleotide minimal substrate and with the structured 58-nucleotide substrate. Multiple turnover and especially the improved accessibility may be very significant

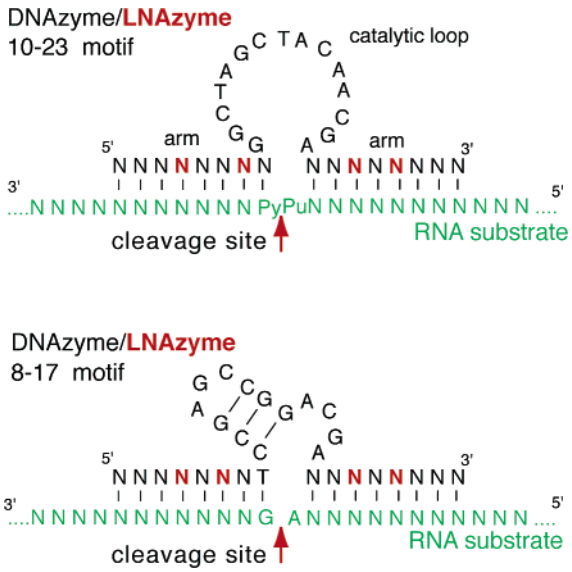


FIGURE 5: Diagrams illustrating the target recognition for 10−23 and 8−17 motif DNA- and LNAzymes. Lines represent Watson−Crick base pairs. The red letters show examples of positioning of LNA monomers for enhanced RNA targeting and cleavage.

for future uses because natural RNAs are usually highly structured. Very similar results were observed by substituting the LNA nucleotides with α-L-LNA nucleotides (*26*). A slightly different approach was used by Schubert *et al*. (*27*), who incorporated three to four LNA monomers at the ends of the binding arms. Although these LNAzymes cleaved well, they were not efficient in multiple turnover, indicating that they bind their RNA target too tightly. It might be very important to carefully adjust the arm length, sequence composition, and number of LNA monomers to optimize RNA cleavage by LNAzymes. In a more recent paper, it is demonstrated how LNAzymes containing three to four LNA monomers at the ends of the binding arms could improve cleavage of unfavorable target structures by improving the target affinity (*28*). Recently, we have extended our LNAzyme analysis to the so-called 8−17 motif DNAzymes (Figure 5), also selected by Santoro and Joyce (*25*), and again we obtained improved performance with incorporation of LNA monomers in the binding arms (our unpublished data). Future research will also be directed toward investigating whether LNA modifications in the catalytic loops are allowed or even advantageous for cleavage. Only a very recent paper has reported on the performance of an LNAzyme transfected into cells. Fahmy *et al*. (*29*) used our LNAzyme design with two LNA monomers dispersed in each binding arm, and they also included a 3′−3′ inverted thymidine monomer at the 3′-end for nuclease stability. They have previously reported inhibition of expression of EGR-1 (early growth response-1) by DNAzyme cleavage (*30*). EGR-1 expression plays a role in the response to injury, resulting in intimal thickening. The recent study (*29*) shows that serum-inducible smooth muscle cell proliferation was inhibited by >50% at LNAzyme concentrations as low as 20 nM, whereas no inhibition by the corresponding DNAzyme was evident at this concentration. They conclude that the LNA-modified DNAzyme is superior at cleaving the EGR-1 transcript, inhibiting endogenous EGR-1 protein expression, SMC proliferation, and regrowth from the wound edge after injury. This study is indeed very promising for a future development of LNAzymes for use in cells and for *in vivo* applications. LNAzymes enhance hybridization, allowing them to access RNA structures not targeted by the corresponding DNAzymes; they show enhanced nuclease stability that increases the half-life in cells, and they allow a multiple-turnover reaction, decreasing the amount of LNAzyme needed.

The above-mentioned way of targeting RNA is only one of many. The term “antisense effect” is widely used and often relates to interference of the processes in which RNA participates from its transcription via mRNA to protein and degradation or as other forms of functional RNA. This includes inhibition or alteration of splicing, translational arrest, and degradation of mRNA. Although the antisense approach seems to be simple, there are a number of hurdles to be overcome like biostability, efficient intracellular delivery, and efficient and specific RNA targeting. By virtue of its intrinsic properties, LNA oligonucleotides are obvious candidates for antisense gene silencing. As many previous LNA antisense studies have already been reviewed (*1*, *2*), only a subset will be mentioned here supplemented with results from very recent studies.

An important and promising *in vivo* antisense experiment with two different LNA sequences targeting DOR mRNA (encoding a receptor) in the central nervous system of living rats was reported (*17*). No tissue damage was observed when the LNA oligonucleotides were injected directly into the brains of living rats, suggesting that acute LNA toxicity is minimal in the living rat brain. Also, a dose-dependent knockdown of DOR was observed with both an LNA/DNA mix-mer and an LNA/DNA/LNA gap-mer, and both LNA oligonucleotides were highly efficacious. In another study, a fully modified LNA targeting the RNA polymerase II gene product inhibited tumor growth *in vivo* in mice and appeared to be nontoxic at <5 mg/kg/day (*31*). The study indicates that LNA is much more potent than the corresponding phosphorothioate−DNA oligonucleotide.

Expression of ICAM-1 (intercellular adhesion molecule 1) was targeted with three different LNA oligomers in a cell line study (*32*). An LNA/DNA gap-mer containing a nine-DNA nucleotide gap potently inhibited ICAM-1 in a dose-dependent manner, and an oligonucleotide with an additional LNA introduced in the middle of the DNA gap was only slightly less potent. Both of these oligomers were shown to be compatible with RNase H activity in *in vitro* assays, and it was assumed that the observed antisense activity was due to an RNase H-dependent mechanism (*32*). Hansen *et al*. (*33*) have reported how incorporation of LNA nucleotides allows shortening of an antisense oligonucleotide for gene silencing. The 20-mer phosphorothioate ISIS 3521 bind to mRNA of human protein kinase C-α and is at present in clinical trials. The oligonucleotide was modified with four LNA monomers at each end, allowing a four-nucleotide shortening without impairment of the repression.

Another example of inhibition by LNA oligonucleotides has been reported with telomerase (*34*), which is responsible for maintaining telomere length from one generation to the next. Telomerase is a ribonucleoprotein that contains a protein domain and RNA with an 11-base sequence that binds telomeric DNA and guides the addition of telomeric repeats. Telomerase is expressed in cancer cells but not in adjoining normal tissue, suggesting that telomerase inhibition will lead

to reduced tumor growth. The RNA moiety of telomerase was targeted with LNA oligonucleotides, and they were found to act as potent and selective inhibitors (*34*). A 13-mer fully modified LNA oligonucleotide and a 13-mer LNA·DNA chimera with three DNA monomers were active with IC_{50} values of 10 nM. The introduction of two terminal phosphorothioate linkages further increased the potency 10-fold but decreased match versus mismatch discrimination. Even very short 8-mer fully modified LNA oligonucleotides were potent inhibitors with IC_{50} values of 2 and 25 nM with and without terminal phosphorothioate linkages, respectively. When the 13-mer LNA oligomers were transfected into cells, an inhibition of more than 80% of the telomerase activity was observed (*34*). Employing short LNA oligonucleotides may cause problems due to binding of nontargeted nucleic acids; however, no alteration of cell morphology was observed within 7 days of transfection of the 8-mer LNAs. Thus, these results suggest that short LNAs may provide not only adequate affinity but also sufficient selectivity for RNA targets in cells.

A different antisense effect has been obtained by using LNA oligonucleotides to inhibit intron splicing. In an *in vitro* study, an 8-mer fully modified LNA and a 12-mer LNA·DNA chimera displayed 50% inhibition at 150 and 30 nM, respectively, of a group I intron splicing in a transcription mixture from *Candida albicans* (*35*). The LNAs were introducing misfolding of the RNA and thereby inhibition of the process of splicing. In another study (*36*), the antisense effects of tricyclo-DNA and LNA oligonucleotides on exon skipping were compared. Nuclear antisense effects of cyclophilin A pre-mRNA splicing by 9−15-mers fully modified oligonucleotides were investigated, and significant inhibition was observed for 11−15-mer tricyclo-DNAs and 13−15-mer LNAs, with tricyclo-DNA being most potent.

Yet another approach to gene silencing is based on siRNA (small interfering RNA duplexes). In the review by Jepsen and Wengel (*2*), LNA oligonucleotides in antisense experiments are evaluated against siRNA, leading to the conclusion that LNA antisense oligonucleotides combined with other modifications such as phosphorothioate linkages might rival the current very popular siRNA approach for gene silencing *in vitro* and *in vivo*. There is one study directly comparing the inhibitory effect of siRNA, LNA/DNA/LNA gap-mers, phosphorothioate DNA, and 2′-*O*-methyl-RNA on the expression of vanilloid receptor subtype 1 in cells (*37*). They found siRNA and LNA/DNA/LNA gap-mers both are very efficient and siRNA is slightly more potent than the LNA/DNA/LNA gap-mers. The siRNAs are composed of approximately 21-nucleotide dsRNA, and they are themselves an obvious choice for incorporation of modified nucleotides for improved biostability and RNA targeting. Although the mechanisms of RNA interference are not fully elucidated, a clearer picture of si/miRNA function and the usefulness of modified nucleotides in siRNA applications is emerging. LNA monomers have been shown to be tolerated in RNAi and to provide thermal stability (*38*). A new finding indicates that weak base pairing at the 5′-end is an important selection criterion for determining which siRNA strand will be used (*39*). Therefore, the exact positioning and the overall number of LNA monomers will be very crucial for optimizing LNA-containing siRNA.

A further example of RNA targeting is on virus genomic RNA. The effects of different LNAs on the interactions of the HIV-1 transactivation responsive element (TAR) have recently been published (*40*). Binding of oligonucleotides to TAR can inhibit Tat-dependent transcription, thereby blocking full-length HIV transcription and hence viral replication. There is a very high degree of sequence conservation in this region of HIV RNA, thus making it an attractive target for drug design. Chimeric sequences of LNA, α-L-LNA, and 2′-thio-LNA (*9*) combined with 2′-*O*-methyl-RNA modifications were investigated, and it was shown that they all inhibited transcription *in vitro* (*40*). The different LNA-type oligonucleotides were transfected into HeLa cells, and intracellular localization and inhibition of transcription were studied by a luciferase reporter assay. Derivatives with a minimum length of 12 residues exhibited 50% inhibition using a nanomolar concentration of LNA oligonucleotides (*40*). This study reveals the potential of LNA oligonucleotides for *in vivo* targeting of RNA using non-RNase H-dependent approaches.

LNA Targeting dsDNA

The term "antigene effect" normally relates to interference with processes that involve chromosomal dsDNA. These include inhibition of transcription, inhibition of replication, and inhibition of other interactions between protein and dsDNA. The task of hybridizing to chromosomal dsDNA that is imbedded in positively charged proteins is obviously not straightforward. Two types of hybridization can be envisaged between dsDNA and single-stranded LNA oligonucleotides, namely, binding to the dsDNA by the formation of a triple-helical complex or binding to the dsDNA by single-strand targeting, thus displacing the other DNA strand by a so-called strand invasion process.

It is envisioned that gene expression can be up- or downregulated at the level of transcription via selective triple-helix formation, preferably at promoter sites, and that triplex-delivered mutagens can modify the genome and homologous recombination can occur through triplex-delivered donor DNA via DNA repair (*41*). These approaches are very appealing, but the lack of affinity of triplex-forming oligonucleotides for their DNA target and sequence constraints imposed by the triple-helical recognition motifs makes it a difficult task. Recent advancements in this area are reviewed by Buchini and Leumann (*41*). Homopyrimidine oligonucleotides are able to target homopurine sequences in dsDNA by Hoogsteen base pairing in the major groove of dsDNA. Thymine forms hydrogen bonds to adenine, and protonated cytosine forms hydrogen bonds to guanine. Triplex formation is strongest at low pH and is relatively weak under physiological conditions. Incorporation of one LNA nucleotide centrally in TFOs (triplex-forming oligonucleotides) significantly increases the binding affinity of the TFOs (*42*). Increasing the number of LNAs in the TFO induced increases in melting temperatures of 4−5 °C per modification at pH 6.6. Also, at pH 7.2, transitions were detectable, demonstrating that triplex formation occurred even at neutral pH. The best results were obtained with TFOs with sequences of alternating DNA and LNA nucleotides, whereas fully modified LNA TFOs showed no propensity at all to hybridize to dsDNA (*43*). Directing an LNA TFO, with alternating DNA and LNA nucleotides, against part of a dsDNA recognized

by the NF-κB transcription factor induced inhibition of binding of NF-κB to the target dsDNA at pH 7.0 by formation of a dsDNA·LNA triplex (*43*). A recent investigation (*44*) of sequence and pH effects of LNA-containing TFOs resulted in the following rules for designing LNA TFO sequences. (i) Choose alternating LNA and DNA nucleotides (e.g., use one LNA every two or three DNA nucleotides). (ii) Maximize the number of LNA 5′−3′ DNA steps (e.g., use one LNA residue at the 5′-end of TFOs when appropriate). (iii) Maximize the number of thymine LNA residues. An *in silicio* analysis of the humane genome has revealed that the population of triplex-forming oligonucleotide sequences is much more abundant than expected from simple random models (*45*). These sequences are often found in regulatory regions, suggesting a good potential for control by triplex formation, and LNAs might be well-suited for such purposes.

Crinelli *et al*. (*23*) have applied various LNA oligomers as decoys for transcription factor NF-κB binding to dsDNA. Inclusion of one or two terminal LNA nucleotides, outside the NF-κB recognition sequence, appreciably improved the protection against nuclease digestion without interfering with transcription factor binding. When LNA nucleotides were included in the NF-κB sequence, further stabilization against degradation was observed. However, these modifications also interfered with NF-κB binding, although in a manner dependent not only on the extent but also on the positioning of the internal LNA substitutions.

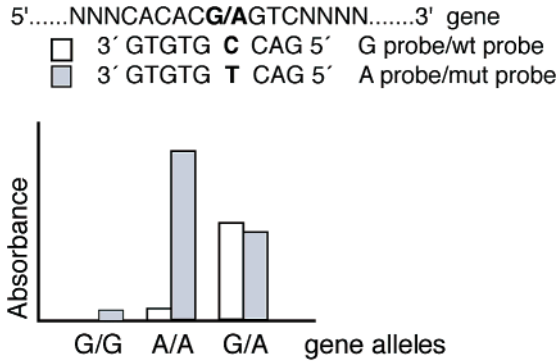
In another approach, LNAs were used to add functionality to plasmid DNA (*46*). LNAs were shown to mediate binding of fluorophores to plasmid DNA, and the LNAs remained associated with plasmid DNA after cationic lipid-mediated transfection into mammalian cells. The mechanism of the binding of LNA oligonucleotides to plasmid DNA was confirmed as being duplex invasion and strand displacement. LNAs were also shown to add CpG adjuvant activity to plasmid DNA without inhibiting high-level antigen expression.

LNAs in Biotechnology Research and Diagnostics

As emphasized above, LNA oligonucleotides display unique hybridization properties and provide decreased susceptibility to nucleases. Both these properties are highly advantageous for a molecular tool for diagnostic applications. The development of such usages of LNA oligonucleotides is likely only in its beginning, and recent examples are described below.

Some heritable genetic defects are caused by single-nucleotide mutations, and there is an increasing demand for detection of these single-nucleotide polymorphisms (SNPs). To screen large populations, simple and cost-effective genotyping arrays are needed. The molecular basis for SNP is to perform PCR on genetic DNA and determine whether the obtained fragments are homozygous or heterozygous with respect to a paticular mutation. Figure 6 illustrates the principle and the specific difference in thermostability between using a DNA or an LNA oligonucleotide for capture. The PCR amplicons are scored by real-time PCR using fluorescent probes or by microtiter plates with an ELISA technique and absorbance determination.

Easy-to-use SNP assays based on the LNA technology have been designed and implemented. LNA is particularly



Thermostability of capture sequence:

Probe	wt-Target	mut-Target
wt-DNA	35 °C	<5 °C
mut-DNA	14 °C	25 °C
wt-LNA	69 °C	34 °C
mut-LNA	29 °C	55 °C

FIGURE 6: Bar graph illustrating the principle of SNP assays. An example of hybridization data is shown below and shows the melting temperatures of the actual capture sequence (*49*). The assay was performed in microtiter plates, and the capture oligonucleotides were connected to the solid support via a 5′-oligonucleotide linker (*49*).

well-suited for this purpose because it leads to a better single-nucleotide mismatch discrimination than that obtained with DNA. For a review on SNP analyses using LNA, see Mouritzen *et al*. (*47*). Several LNA genotyping assays have been reported, including screening for an apolipoprotein B (apoB) R3500Q mutation and two mutations in apolipoprotein E (*48*, *49*) and for the factor V Leiden mutation (*50*). ApoB-100 is an important determinant of LDL cholesterol in plasma, and the R3500Q mutation reduces the affinity for the LDL receptor and hence elevates the level of LDL cholesterol in plasma. The apoB R3500Q mutation is the major cause of familial defective apoB-100, which increases the risk of coronary heart disease. Carriers of the factor V Leiden mutation have an increased risk of venous thromboembolism. The use of LNAs relative to DNA in the detection of the factor V Leiden mutation and the sickle cell anemia mutation in the β-globin gene were compared in 5′-nuclease assays using phosphorothioate antisense oligonucleotide hybridization. LNA oligonucleotides were shown to have an enhanced discriminatory power, outperforming the comparable DNA probe (*51*). Also, the use of LNAs in 5′-nuclease assays compared to routinely used MGB (minor groove binder) probes was evaluated (*52*). The 5′-nuclease PCR is based on the use of a fluorogenic probe that hybridizes within the DNA target sequence bound by PCR primers. The reporter fluorescence is suppressed when the probe is intact because of the proximity of a quencher. During PCR, the 5′-nuclease activity of AmpliTaq Gold DNA polymerase cleaves the probe, resulting in the increased fluorescence of the reporter. The sensitivity and specificity of 5′-nuclease PCR assays targeting staphylococcal enterotoxin genes were compared and showed that the LNA and MGB methods were equivalent. The conclusion was that PCR combined with LNA probes proved to be a promising alternative to MGB probes for rapid detection of genes of bacterial pathogens (*52*).

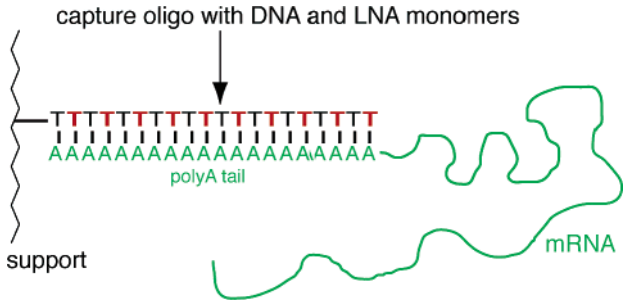


FIGURE 7: Sketch of an example of LNA used as an mRNA capture probe (*53*).

Another application of LNA is as a capture probe for isolating mRNA with polyA sequences in the 3′-end (*53*) (Figure 7). A 20-mer polyT probe with alternating LNA and DNA monomers increased the mRNA yield by 30−50-fold compared to a 20-mer polyT DNA. The extraction was also efficient in low-salt buffers and offers an attractive alternative to current methods (*53*). The use of LNA in microarrays has also been exploited. An optimal design for LNAs as capture probes for gene expression profiling together with a microarray study of *Caenorhabditis elegans* cytochrome expression has been published (*54*). The oligo design program, which might be useful in many applications, is freely available at http://lnatools.com/.

LNA oligonucleotides have furthermore been successfully applied to FISH (fluorescence *in situ* hybridization) (*55*). The optimal design of LNA/DNA mix-mers and buffer conditions for targeting a 23 bp satellite sequence were investigated. The LNA/DNA mix-mers were 5′-labeled with Cy3 or biotin and targeted to human chromosome preparations. Mix-mers with every second nucleotide modified gave better results than mix-mers with every third nucleotide modified. A strong signal was obtained in a short time compared to that with DNA oligonucleotides, and a signal was obtained even without denaturation (*55*).

Very recently, an LNA-based oligonucleotide set of 90 sequences has become commercially available for "transcripsome investigation" of human and mouse cells (http://www.ProbeLibrary.com). The high affinity provided by LNA allows short sequences of eight to nine bases to hybridize to PCR products, and a set of only 90 oligos can therefore target all transcripts in the cells. Specific primers ensure the specificity of the real-time PCR reaction, whereas the LNA oligonucleotide probes provide the fluorescence signal for quantification.

Future Aspects

LNAs constitute an important addition to the tools available for biotechnology, nucleic acid diagnostics, and nucleic acid therapeutics. The remarkable hybridization properties of LNA, with respect to both affinity and specificity, make it a technology-improving, or even technology-enabling, molecule for molecular biology research and biotechnology innovation. The facts that LNA phosphoramidites and oligomers are commercially available and that LNA nucleotides can be freely mixed with DNA, RNA, and 2′-*O*-Me-RNA monomers and standard probes make LNA a very flexible tool, allowing fine-tuning of properties.

Short LNA oligonucleotides (fully modified) and short LNA/DNA and LNA/RNA mix-mer oligonucleotides are uniquely suited for targeting of complementary RNA and DNA. Especially with respect to *in vitro* and *in vivo* RNA analysis and targeting, LNA will likely have significant impact. LNA rivals siRNA for gene silencing, but a satisfactory answer to the demand for efficient cellular delivery *in vivo* remains to be developed for LNA, as for any other therapeutic oligonucleotide that relies on hybridization to a nucleic acid target for biological activity. α-L-LNA is a promising LNA stereoisomer, likewise displaying excellent hybridization properties, that has shown a relatively good ability to support RNase H activity and excellent nuclease resistance. 2′-Amino-LNA can be equipped with various functional groups without compromising the LNA-type high-affinity hybridization capabilities (*56*). Research in these directions will pave the way for exploratory uses of oligonucleotides, including LNA and functionalized LNA, in the emerging areas of nanotechnology and aptamer architecture. One example is the incorporation of LNA in quadruplex structures, which may find use in determining the biological significance of G-quadruplex folding topology, and controlling G-quadruplex folding for applications in nanotechnology (*57*). Simple molecular-scale autonomous programmable computers based on nucleic acid interactions have been demonstrated, and a vision is a nucleic acid-based autonomous biomolecular computer that logically analyzes the levels of messenger RNA species, and in response produces a molecule capable of affecting levels of gene expression (*58*). The superb hybridization properties of LNA allowing shortening of target sequences and its nuclease stability could make LNA an important partner in such designs. LNA is a unique molecule, which is likely to make a significant impact on the future developments within many areas of biotechnology, as should be evident from all the descriptions presented above.

REFERENCES

1. Petersen, M., and Wengel, J. (2003) LNA: a versatile tool for therapeutics and genomics, *Trends Biotechnol. 21*, 74−81.
2. Jepsen, J. S., and Wengel, J. (2004) Locked Nucleic Acid: a potent nucleic acid analogue in therapeutics and biotechnology, *Curr. Opin. Drug Discovery Dev. 7*, 188−194.
3. Singh, S. K., Nielsen, P., Koshkin, A. A., and Wengel, J. (1998) LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition, *Chem. Commun. 4*, 455−456.
4. Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E., and Wengel, J. (1998) LNA (Locked Nucleic Acids): Synthesis of the Adenine, Cytosine, Guanine, 5-Methylcytosine, Thymine and Uracil Bicyclonucleoside Monomers, Oligomerisation, and Unprecedented Nucleic Acid Recognition, *Tetrahedron 54*, 3607−3630.
5. Obika, S., Nanbu, D., Hari, Y., Morio, K., In, Y., Ishida, T., and Imanishi, T. (1997) Synthesis of 2′-*O*,4′-*C*-methyleneuridine and -cytidine novel bicyclic nucleosides having a fixed C3′-*endo* sugar puckering, *Tetrahedron Lett. 38*, 8735−8738.
6. Obika, S., Nanbu, D., Hari, Y., Andoh, J., Morio, K., Doi, T., and Imanishi, T. (1998) Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2′-O,4′-C-methyleneribonucleosides, *Tetrahedron Lett. 39*, 5401−5404.
7. Sørensen, M. D., Kvaernø, L., Bryld, T., Håkansson, A. E., Verbeure, B., Gaubert, G., Herdewijn, P., and Wengel, J. (2002) α-L-*ribo*-Configured locked nucleic acid (α-L-LNA): synthesis and properties, *J. Am. Chem. Soc. 124*, 2164−2176.
8. Wengel, J. (1999) Synthesis of 3′-*C*- and 4′-*C*-branched oligonucleotides and the development of locked nucleic acid (LNA), *Acc. Chem. Res. 32*, 301−310.
9. Kumar, R., Singh, S. K., Koshkin, A. A., Rajwanshi, V. K., Meldgaard, M., and Wengel, J. (1998) The first analogues of LNA

(locked nucleic acids): Phosphorothioate-LNA and 2′-thio-LNA, *Biorg. Med. Chem. Lett. 8*, 2219−2222.
10. McTigue, P. M., Peterson, R. J., and Kahn, J. D. (2004) Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation, *Biochemistry 43*, 5388−5405.
11. Petersen, M., Bondensgaard, K., Wengel, J., and Jacobsen, J. P. (2002) Locked nucleic acid (LNA) recognition of RNA: NMR solution structures of LNA:RNA hybrids, *J. Am. Chem. Soc. 124*, 5974−5982.
12. Nielsen, K. E., Rasmussen, J., Kumar, R., Wengel, J., Jacobsen, J. P., and Petersen, M. (2004) NMR Studies of Fully Modified Locked Nucleic Acid (LNA) Hybrids: Solution Structure of an LNA:RNA Hybrid and Characterization of an LNA:DNA Hybrid, *Bioconjugate Chem. 15*, 449−457.
13. Petersen, M., Nielsen, C. B., Nielsen, K. E., Jensen, G. A., Bondensgaard, K., Singh, S. K., Rajwanshi, V. K., Koshkin, A. A., Dahl, B. M., Wengel, J., and Jacobsen, J. P. (2000) The conformations of locked nucleic acids (LNA), *J. Mol. Recognit. 13*, 44−53.
14. . Nielsen, K. M. E., Petersen, M., Håkansson, A. E., Wengel, J., and Jacobsen, J. P. (2002) α-L-LNA (α-L-ribo Configured Locked Nucleic Acid) Recognition of DNA: An NMR Spectroscopic Study, *Chem.−Eur. J. 8*, 3001−3009.
15. Nielsen, J. T., Stein, P. C., and Petersen, M. (2003) NMR structure of an α-L-LNA:RNA hybrid: structural implications for RNase H recognition, *Nucleic Acids Res. 31*, 5858−5867.
16. Fedoroff, O. Y., Salazar, M., and Reid, B. R. (1993) Structure of a DNA:RNA hybrid duplex. Why RNase H does not cleave pure DNA, *J. Mol. Biol. 233*, 509−523.
17. . Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hökfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K., Ossipov, M., Koshkin, A., Jakobsen, N., Skouv, J., Oerum, H., Jacobsen, M. H., and Wengel, J. (2000) Potent and nontoxic antisense oligonucleotides containing locked nucleic acids, *Proc. Natl. Acad. Sci. U.S.A. 97*, 5633−5638.
18. . Kurreck, J., Wyszko, E., Gillen, C., and Erdmann, V. A. (2002) Design of antisense oligonucleotides stabilized by locked nucleic acids, *Nucleic Acids Res. 30*, 1911−1918.
19. Frieden, M., Christensen, S. M., Mikkelsen, N. D., Rosenbohm, C., Thrue, C. A., Westergaard, M., Hansen, H. F., Orum, H., and Koch, T. (2003) Expanding the design horizon of antisense oligonucleotides with α-L-LNA, *Nucleic Acids Res. 31*, 6365−6372.
20. Frieden, M., Hansen, H. F., and Koch, T. (2003) Nuclease stability of LNA oligonucleotides and LNA-DNA chimeras, *Nucleosides, Nucleotides Nucleic Acids 22*, 1041−1043.
21. Morita, K., Hasegawa, C., Kaneko, M., Tsutsumi, S., Sone, J., Ishikawa, T., Imanishi, T., and Koizumia, M. (2002) 2′-*O*,4′-*C*-Ethylene-Bridged Nucleic Acids (ENA): Highly Nuclease-Resistant and Thermodynamically Stable Oligonucleotides for Antisense Drug, *Bioorg. Med. Chem. Lett. 12*, 73−76.
22. Lauritsen, A., Dahl, B. M., Dahl, O., Vester, B., and Wengel, J. (2003) Methylphosphonate LNA: a locked nucleic acid with a methylphosphonate linkage, *Bioorg. Med. Chem. Lett. 13*, 253−256.
23. Crinelli, R., Bianchi, M., Gentilini, L., and Magnani, M. (2002) Design and characterization of decoy oligonucleotides containing locked nucleic acids, *Nucleic Acids Res. 30*, 2435−2443.
24. Di Giusto, D. A., and King, G. C. (2004) Strong positional preference in the interaction of LNA oligonucleotides with DNA polymerase and proofreading exonuclease activities: implications for genotyping assays, *Nucleic Acids Res. 32*, e32.
25. Santoro, S. W., and Joyce, G. F. (1997) A general purpose RNA-cleaving DNA enzyme, *Proc. Natl. Acad. Sci. U.S.A. 94*, 4262−4266.
26. Vester, B., Lundberg, L. B., Sørensen, M. D., Babu, B. R., Douthwaite, S., and Wengel, J. (2002) LNAzymes: incorporation of LNA-type monomers into DNAzymes markedly increases RNA cleavage, *J. Am. Chem. Soc. 124*, 13682−13683.
27. Schubert, S., Gul, D. C., Grunert, H. P., Zeichhardt, H., Erdmann, V. A., and Kurreck, J. (2003) RNA cleaving "10−23" DNAzymes with enhanced stability and activity, *Nucleic Acids Res. 31*, 5982−5992.
28. Schubert, S., Fürste, J. P., Werk, D., Grunert, H.-P., Zeichhardt, H., Erdmann, V. A., and Kurreck, J. (2004) Gaining target access for deoxyribozymes, *J. Mol. Biol. 339*, 355−363.
29. Fahmy, R. G., and Khachigian, L. M. (2004) Locked nucleic acid modified DNA enzymes targeting early growth response-1 inhibit human vascular smooth muscle cell growth, *Nucleic Acids Res. 32*, 2281−2285.
30. Khachigian, L. M., Fahmy, R. G., Zhang, G., Bobryshev, Y. V., and Kaniaros, A. (2002) c-Jun regulates vascular smooth muscle cell growth and neointima formation after arterial injury. Inhibition by a novel DNA enzyme targeting c-Jun, *J. Biol. Chem. 277*, 22985−22991.
31. Fluiter, K., ten Asbroek, L. M. A., de Wissel, M. B., Jakobs, M. E., Wissenbach, M., Olsson, H., Olsen, O., Ørum, H., and Baas, F. (2003) In vivo tumor growth inhibition and biodistribution studies of locked nucleic acid (LNA) antisense oligonucleotides, *Nucleic Acids Res. 31*, 953−962.
32. Obika, S., Hemamayi, R., Masuda, T., Sugimoto, T., Nakagawa, S., Mayumi, T., and Imanishi, T. (2001) Inhibition of ICAM-1 gene expression by antisense 2′,4′-BNA oligonucleotides, *Nucleic Acids Res. Suppl.*, 145−146.
33. Hansen, J. B., Westergaard, M., Thrue, C. A., Giwercman, B., and Oerum, H. (2003) Antisense knockdown of PKC-α using LNA-oligos, *Nucleosides, Nucleotides Nucleic Acids 22*, 1607−1609.
34. Elayadi, A. N., Braasch, D. A., and Corey, D. R. (2002) Implications of high-affinity hybridization by locked nucleic acid oligomers for inhibition of human telomerase, *Biochemistry 41*, 9973−9981.
35. Childs, J. L., Disney, M. D., and Turner, D. H. (2002) Oligonucleotide directed misfolding of RNA inhibits *Candida albicans* group I intron splicing, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11091−11096.
36. Ittig, D., Liu, S., Renneberg, D., Schumperli, D., and Leumann, C. J. (2004) Nuclear antisense effects in cyclophilin A pre-mRNA splicing by oligonucleotides: a comparison of tricyclo-DNA with LNA, *Nucleic Acids Res. 32*, 346−353.
37. Grünweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and small interfering RNA, *Nucleic Acids Res. 31*, 3185−3193.
38. Braasch, D. A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M. A., and Corey, D. R. (2003) RNA interference in mammalian cells by chemically-modified RNA, *Biochemistry 42*, 7967−7975.
39. Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003) Asymmetry in the Assembly of the RNAi Enzyme Complex, *Cell 115*, 199−208.
40. Arzumanov, A., Stetsenko, D. A., Malakhov, A. D., Reichelt, S., Sorensen, M. D., Babu, B. R., Wengel, J., and Gait, M. J. (2003) A structure−activity study of the inhibition of HIV-1 Tat-dependent trans-activation by mixmer 2′-O-methyl oligoribonucleotides containing locked nucleic acid (LNA), α-L-LNA, or 2′-thio-LNA residues, *Oligonucleotides 13*, 435−453.
41. Buchini, S., and Leumann, C. J. (2003) Recent improvements in antigene technology, *Curr. Opin. Chem. Biol. 7*, 717−726.
42. Obika, S., Hari, Y., Sugimoto, T., Sekiguchi, M., and Imanishi, T. (2000) Triplex-forming enhancement with high sequence selectivity by single 2′-*O*,4′-*C*-methylene bridged nucleic acid (2′,4′-BNA) modification, *Tetrahedron Lett. 41*, 8923−8927.
43. Obika, S., Uneda, T., Sugimoto, T., Nanbu, D., Minami, T., Doi, T., and Imanishi, T. (2001) 2′-*O*,4′-*C*-Methylene bridged nucleic acid (2′,4′-BNA): synthesis and triplex-forming properties, *Bioorg. Med. Chem. 9*, 1001−1011.
44. Sun, B. W., Babu, B. R., Sørensen, M. D., Zakrzewska, K., Wengel, J., and Sun, J. S. (2004) Sequence and pH effects of LNA-containing triple helix-forming oligonucleotides: physical chemistry, biochemistry, and modeling studies, *Biochemistry 43*, 4160−4169.
45. Goni, J. R., de la Cruz, X., and Orozco, M. (2004) Triplex-forming oligonucleotide target sequences in the human genome, *Nucleic Acids Res. 32*, 354−360.
46. Hertoghs, K. M., Ellis, J. H., and Catchpole, I. R. (2003) Use of locked nucleic acid oligonucleotides to add functionality to plasmid DNA, *Nucleic Acids Res. 31*, 5817−5830.
47. Mouritzen, P., Nielsen, A. T., Pfundheller, H. M., Choleva, Y., Kongsbak, L., and Møller, S. (2003) Single nucleotide polymorphism genotyping using locked nucleic acid (LNA), *Expert Rev. Mol. Diagn. 3*, 27−38.
48. Jacobsen, N., Bentzen, J., Meldgaard, M., Jakobsen, M. H., Fenger, M., Kauppinen, S., and Skouv, J. (2002) LNA-enhanced detection of single nucleotide polymorphisms in the apolipoprotein E, *Nucleic Acids Res. 30*, e100.

49. Jacobsen, N., Fenger, M., Bentzen, J., Rasmussen, S. L., Jakobsen, M. H., Fenstholt, J., and Skouv, J. (2002) Genotyping of the apolipoprotein B R3500Q mutation using immobilized locked nucleic acid capture probes, *Clin. Chem. 48*, 657−660.
50. Ørum, H., Jakobsen, M. H., Koch, T., Vuust, J., and Borre, M. B. (1999) Detection of the factor V Leiden mutation by direct allele-specific hybridization of PCR amplicons to photoimmobilized locked nucleic acids, *Clin. Chem. 45*, 1898−1905.
51. Ugozzoli, L. A., Latorra, D., Pucket, R., Arar, K., and Hamby, K. (2004) Real-time genotyping with oligonucleotide probes containing locked nucleic acids, *Anal. Biochem. 324*, 143−152.
52. Letertre, C., Perelle, S., Dilasser, F., Arar, K., and Fach, P. (2003) Evaluation of the performance of LNA and MGB probes in 5′-nuclease PCR assays, *Mol. Cell Probes 17*, 307−311.
53. Jacobsen, N., Nielsen, P. S., Jeffares, D. C., Eriksen, J., Ohlsson, H., Arctander, P., and Kauppinen, S. (2004) Direct isolation of poly(A)$^+$ RNA from 4 M guanidine thiocyanate-lysed cell extracts using locked nucleic acid-oligo(T) capture, *Nucleic Acids Res. 32*, e64.
54. Tolstrup, N., Nielsen, P. S., Kolberg, J. G., Frankel, A. M., Vissing, H., and Kauppinen, S. (2003) OligoDesign: Optimal design of LNA (locked nucleic acid) oligonucleotide capture probes for gene expression profiling, *Nucleic Acids Res. 31*, 3758−3762.
55. Silahtaroglu, A. N., Tommerup, N., and Vissing, H. (2003) FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers, *Mol. Cell Probes 17*, 165−169.
56. Sørensen, M. D., Petersen, M., and Wengel, J. (2003) Functionalized LNA (locked nucleic acid): high-affinity hybridization of oligonucleotides containing N-acylated and N-alkylated 2′-amino-LNA monomers, *Chem. Commun. 17*, 2130−2131.
57. Dominick, P. K., and Jarstfer, M. B. (2004) A Conformationally Constrained Nucleotide Analogue Controls the Folding Topology of a DNA G-Quadruplex, *J. Am. Chem. Soc. 126*, 5050−5051.
58. Benenson, Y., Gil, B., Ben-Dor, U., Adar, R., and Shapiro, E. (2004) An autonomous molecular computer for logical control of gene expression, *Nature 429*, 423−429.

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