# CHAPTER 9

# LOCKED NUCLEIC ACID OLIGONUCLEOTIDES TOWARD CLINICAL APPLICATIONS

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## 9.1. INTRODUCTION

Nucleic acid–based therapeutic technologies (Figure 9.1) have significantly advanced in the past two decades toward the treatment of many diseases. The first such drug to enter clinic was vitravene®, an antisense oligonucleotide for the treatment of cytomegalovirus retinitis [1]. Later, research on aptamers led to the marketing of macugen®, an inhibitor of vascular endothelial growth factor (VEGF) for the treatment of age related macular degeneration (AMD) [2]. Nucleic acid–based therapeutic approaches mainly include antisense [3,4], ribozymes [4], small interfering RNA (siRNA) [4–6], microRNA (miRNA) [7–10] targeting and aptamers [11–15]. Oligonucleotides composed of naturally occurring DNA or RNA nucleotides pose some limitations because of their poor RNA binding affinity, low degree of nuclease resistance, and low bioavilability. To overcome these limitations, chemically modified nucleic acids have been introduced, among which locked nucleic acid (LNA) [16–20] proved to be unique and is now used extensively for various applications in chemical biology [21–23].

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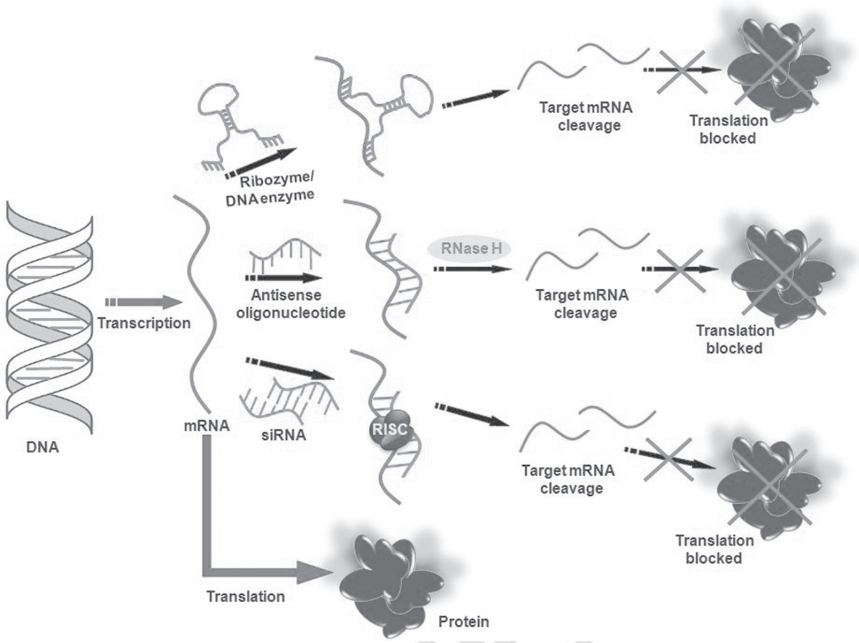


**Figure 9.1** Schematic illustration of various nucleic acid gene silencing techniques. *(Full-color version of the figure appears in the color plate section.)*

## 9.2. LOCKED NUCLEIC ACID (LNA)

LNA nucleotides are generally considered to be RNA mimicking molecule in which the ribose sugar moiety is locked by an oxymethylene bridge connecting the C2′ and C4′ carbon atoms, imposing conformational restriction to adopt C3′-endo/*N* -type furanose conformation (Figure 9.2) [16,19,24]. Structural investigation by NMR spectroscopy has shown that LNA-containing oligonucleotides tend to adopt A-type duplex geometris [25,26]. Commercially available LNA contains natural phosphodiester linkages and therefore resembles natural nucleic acids in terms of aqueous solubility, Watson-Crick mode of binding, and straightforward automated synthesis using standard phosphoramidite chemistry.

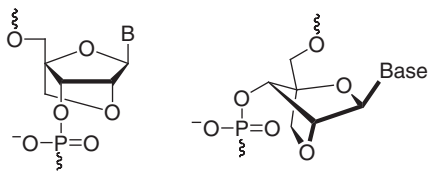


**Figure 9.2** Structural representation of LNA monomers.

**TABLE 9.1 Examples of melting temperatures ($T_m$ values) for hybridization of LNA and DNA oligonucleotides to complementary DNA and RNA oligonucleotide sequences. (Data collected from Singh *et al*. [12]). LNA modifications are represented in bold underlined capital letters; DNA monomers in capital letters; RNA monomers are in capital italic letters**

| DNA/LNA: DNA/*RNA* duplexes | Melting temperatures (°C) |
|---|---|
| 5′-**TTTTTT: AAAAAA** | <10 |
| 5′-**TTTTTT: AAAAAA** | 32 |
| 5′-**TTTTTT: AAAAAA** | <10 |
| 5′-**TTTTTT: AAAAAA** | 40 |
| 5′-**GTGATATGC: CACTATACG** | 28 |
| 5′-**GTGATATGC: CACTATACG** | 44 |
| 5′-**GTGATATGC: CACUAUACG** | 28 |
| 5′-**GTGATATGC: CACUAUACG** | 50 |

LNA offers unique properties needed for successful therapeutic application of oligonucleotides. First and most important, LNA oligonucleotides possess extremely high binding affinity to complementary DNA and RNA oligonucleotides as evidenced by thermal denaturation studies, that is, an increase in melting temperature ($T_m$) of +2 to +8°C per LNA monomer compared to the unmodified duplexes [16–20]. It is also noted that this increase in affinity goes hand in hand with preserved, or even improved, Watson-Crick base-pairing selectivity. Some examples of melting temperatures for LANs hybridized to RNA are listed in Table 9.1. LNA:LNA base paring is also very strong [17] and should be considered in relation to the risk of self-complementarity when designing LNAs for biological experiments (for LNA design tools. visit www.exiqon.com). In addition to increased binding affinity, properly designed LNA oligonucleotides display improved mismatch discrimination (or improved Watson-Crick base-pairing selectivity) relative to unmodified nucleic acids, and high stability in biological systems (i.e., resistance toward enzymatic degradation) [22,23].

### 9.2.1. Therapeutic Applications of LNA

For effective modulation of gene expression, the advantages of synthetic oligonucleotides are exploited through binding of an antisense oligonucleotide (AON) to a specific mRNA or noncoding RNA by Watson-Crick base pairing. Upon binding, the oligonucleotide can modulate RNA processing and inhibit translation, induce splice switching, or promote degradation. Various such techniques include classical antisense, siRNA, antimiRs (antagomirs), and DNAzymes. The usefulness of LNA-modified oligonucleotides in gene silencing techniques (Figure 9.3) has been the subject for many scientific investigations, and successful studies on LNA oligonucleotides have already been the topic of detailed reviews [27–29].

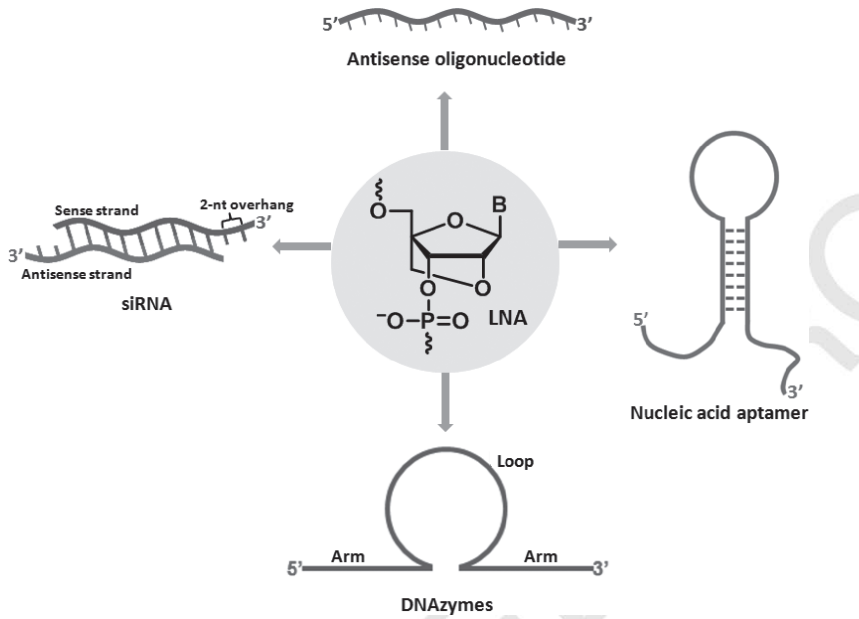


**Figure 9.3** Examples of various applications of LNA technology. LNA nucleotides can be incorporated at desired sites in these constructs.

### 9.2.2. LNA Antisense

The first- and second-generation antisense oligonucleotides (AsONs) have been evolved to contain DNA phophorothioate nucleotides coupled with other chemical modifications such as 2′-*O*-Methyl-RNA (2′-OMe) and 2′-*O*-Methoxyethyl-RNA (2′-MOE) [30]. The latest generation of AsONs includes LNA, which shows exceptionally high binding affinity to RNA and nuclease stability. This might account for low nanomolar or high picomolar $IC_{50}$ values for mRNA down-regulation that have generally been achieved in cell-culture for LNA-AsONs [31,32]. The majority of the antisense experiments made with LNA have been focused on mRNA inhibition by RNase H recruitment, although non-RNase H mechanisms have also been reported. Wahlestedt *et al*., [33] first reported the effect of LNA as an antisense molecule by demonstrating the feasibility of LNA to act as potent and nontoxic nucleic acid modification *in vivo*. The experiments involved two different types of LNA AsONs, namely LNA/DNA mixmer and LNA/DNA/LNA gapmer constructs (Figure 9.4), targeting the delta opioid receptor (DOR) mRNA in the central nervous system of rats. An efficient knockdown of DOR was induced with both types on direct injection of the LNA AsONs into the rat brain. A number of reports have subsequently highlighted the broad potential of LNA-modified oligonucleotides in antisense mediated gene silencing applications *in vitro* and *in vivo* [27–29]. A recent report from Jacobsen *et al*. [34], for example, showed that LNA AONs

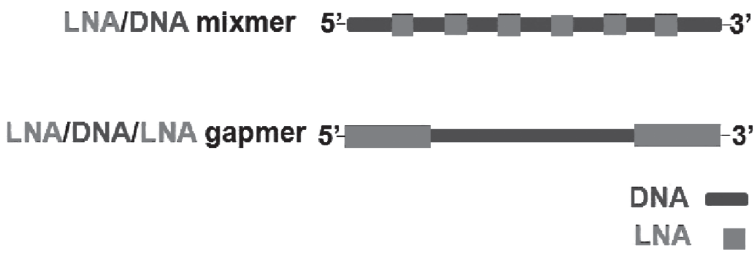


**Figure 9.4** Design of LNA AsONs; mixmer and gapmer constructs.

are effective inhibitors of HIV-1 expression. Another recent report by Sapra *et al*., [35] demonstrated efficient down-regulation of survivin expression thereby inhibiting tumor growth *in vivo* using an LNA AsON. Santaris Pharma and its partner, Enzon Pharmaceuticals, have advanced three LNA AsONs-based drugs into clinical trials. Each of these LNA-based drug candidates targets mRNAs that are fundamental to cancer biology, enabling them, if successful, to be used in the treatment of solid and nonsolid tumors.

Stein *et al*., [37] recently developed a method called "gymnosis" to deliver LNA AsONs to the target and does not require any transfection reagents or any additives to serum as the oligomers were delivered naked. This technique takes advantage of the normal growth properties of cells in tissue culture to promote productive oligonucleotide uptake and permits sequence-specific silencing of multiple targets in a large number of cell types in tissue culture at low micromolar concentrations. Using this approach, they targeted a 16-mer AsON containing phosphorothioates and LNA nucleotides to the codons 1–6 of the Bcl-2 mRNA using 518A2 melonoma cells. They observed substatial silencing of Bcl-2 protein expression. However, also note that gymnotic silencing is relatively slow compared to lipofection-mediated silencing.

### 9.2.3. LNAzymes (LNA-Modified DNAzymes)

DNAzymes are catalytically active DNA molecules that are able to cleave RNA in a sequence-specific manner after hybridization, thereby functioning as a specific RNA endonuclease by cleaving the phosphodiester backbone of the RNA target [38,39]. Studies conducted with LNA-modified DNAzymes, termed LNAzymes, showed an enhanced efficiency of RNA cleavage [39,40]. In this direction, Vester *et al*. [40] investigated the effect of LNA by incorporating two LNA nucleotides in each of the binding arms of the DNAzyme, which yielded an LNAzyme with a highly enhanced efficiency of RNA cleavage. The experiment demonstrated cleavage of highly structured targets (a 58n long RNA with known secondary structure and 2904n long 23S ribosomal RNA subunit), which was significantly improved using LNAzymes instead of the corresponding unmodified DNAzymes. A similar approach was employed by Schubert *et al*. [41] who incorporated 3–4 LNA monomers at the ends of the binding arms and observed a highly enhanced

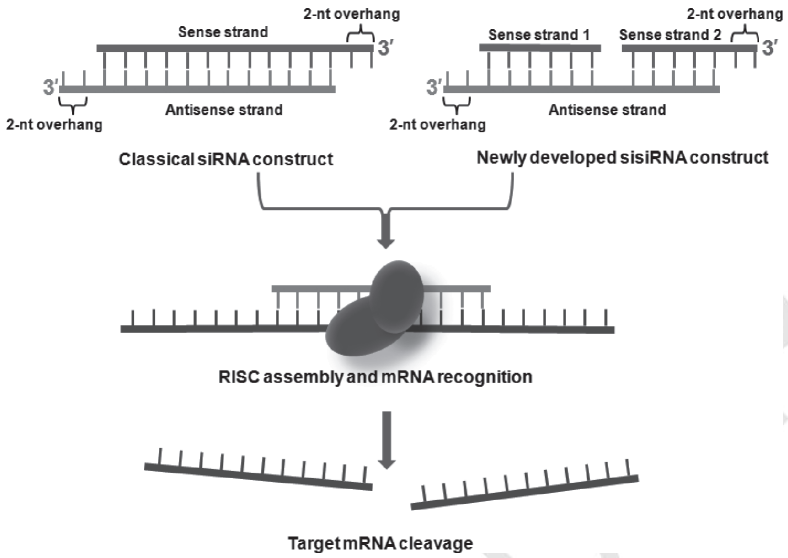


**Figure 9.5** Schematic illustration of siRNA- and sisiRNA-based gene silencing approach.

efficiency of RNA cleavage. In addition, they later demonstrated that LNAzymes containing 3–4 LNA monomers at the ends of the binding arms can cleave viral RNA structures that are resistant to cleavage by the corresponding unmodified DNAzymes [42]. More recently, Jacobsen *et al*., reported efficient inhibition of HIV-1 expression by targeting LNAzymes to functionally selected binding sites [36], whereas targeting of miRNAs by using LNAzymes was recently been reported by Jadhav *et al*. [43]. No LNAzymes have entered preclinical drug development.

### 9.2.4. LNA in RNA Interference (siLNA)

RNA interference (RNAi) has emerged as a powerful approach to knock down gene expression [44]. In this method, double-stranded small interfering RNA (siRNA) constructs are used to target mRNA and induce its degradation on incorporation of the so-called antisense strand into the RNA-induced silencing complex (RISC; Figure 9.5). Chemical modification can be introduced in synthetic siRNA candidates for improved biostability, pharmacokinetics, and effective RNA targeting. In this approach, the application of LNA-modified siRNA, termed siLNA, has been investigated.

Braasch *et al*. [45]. first conducted a study in mammalian cells using LNA-modified siRNA (siLNA) and observed that the introduction of LNA nucleotides substantially increased the thermal stability of the modified RNA duplex without compromising the efficiency of RNAi. Another study by Elmén *et al*. [46]. showed that LNA-modified siRNA duplexes display remarkably enhanced serum

half-life compared to the corresponding unmodified siRNAs. The report also highlighted an improved efficacy of LNA-modified variants on certain RNA motifs relevant for targeting the SARS-CoV virus, in addition to providing evidence that the use of siLNAs reduces sequence-related off-target effects. Mook *et al*. [47]. Evaluated the effect of LNA-modified siRNA both *in vitro* and *in vivo*. They positioned two LNA-T nucleotides at the 3′-end of the sense and antisense strand, and the sense strand was further modified by single or multiple LNA nucleotides at the 5′-end. They showed that minimal LNA-modifications at the 3′-end of siRNA are effective to stabilize siRNA, and that multiple LNA modifications may lead to decreased efficacy *in vitro* and *in vivo*. The study further revealed a reduced off-target gene regulation when using LNA-modified siRNA compared to the unmodified siRNA. Very recently, Bramsen *et al*., introduced a three-stranded siRNA construct termed "small internally segmented interfering RNA" (sisiRNA), in which the antisense strand is complexed with two short sense strands of approximately 10–12 nt in length [48]. In the sisiRNA approach, only the antisense strand is functional as the nick completely eliminates unintended mRNA targeting by the sense strand (Figure 9.5). LNA nucleotides were incorporated to stabilize the sisiRNA constructs, which proved efficient for gene silencing on transfection into an H1299 lung carcinoma cell line [48]. A recent report by Laursen *et al*. [49] shows that the destabilizing properties of unlocked nucleic acids (UNA) can be applied to enhance the potency of siRNAs, which are heavily modified by LNA. No siLNA construct has entered preclinical drug development.

### 9.2.5. LNA Probes for MicroRNA Targeting

MicroRNAs (miRNAs) are short, typically 19- to 25-nt long, endogenous noncoding RNAs that are processed from longer hairpin transcripts by the enzyme Dicer [7–9]. MicroRNAs repress the expression of protein-coding genes posttranscriptionally by hybridizing with the 3′-untranslated regions (UTRs) of the target messenger RNAs, and emerging evidence demonstrates that miRNAs present in animals are important in controlling many biological processes [7–9]. Similar to classical AsONs developed for the inhibition of coding RNAs, synthetic oligonucleotides (antimiRs) are the only rational approach for specific inhibition of the individual miRNAs and therefore have the potential to be developed as an important new class of drugs. In this direction, the application of LNA modifications has been investigated by Válóczi *et al*. [50], who described an efficient detection of miRNAs by Northern blot analysis using LNA-modified oligonucleotide probes and demonstrated their improved sensitivity to detect different miRNAs in animals and plants. The probes were between 20 and 23 nt long and modified with seven LNA nucleotides at variuos positions from 5′- to 3′-end. Wienholds *et al*. [51]. determined the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebra fish embryos by *in situ* hybridizations using LNA-modified oligonucleotide probes. Another report highlighted a sensitive microarray platform using LNA-modified capture probes

for miRNA expression profiling [52]. Elmén *et al*. [53] described that effective LNA antimiRs (LNA/DNA mixmer olionucleotides) mediated miRNA silencing in nonhuman primates of liver-expressed miR-122. Their findings demonstrate the potential of these compounds as a new class of potential therapeutics for disease associated with miRNAs. Fabani *et al*. [54]. showed that lipofection of an antisense oligonucleotide based on a LNA/2′-*O*-methyl RNA mixmer oligomer is effective at blocking miR-122 activity in human and rat liver cells. Another recent report demonstrates an effective silencing of liver expressed miR-122 in primates with chronic hepatitis C virus (HCV) infection [10]. They show that an LNA-modified oligonucleotide (SPC 3649) complementary to miR-122 successfully inhibits miR-122, a microRNA important for hepatitis C viral replication, thereby significantly reducing hepatitis C virus in the bloodstream in chimpanzees chronically infected with the hepatitis C virus. This LNA antimir (SPC3649) is the first microRNA-targeted drug to enter human clinical trials, as the Phase 1 clinical trial is ongoing [36].

### 9.2.6. LNA Aptamers

Aptamers [11–15] are short DNA or RNA oligonucleotide sequences that can bind to their targets with high affinity and specificity because of their ability to adopt three-dimensional structures. Aptamers are generated by a process referred to as SELEX (Systematic Evolution of Ligands by Exponential enrichment) [55–58]. The remarkable properties and applications of LNA highlighted earlier, particularly their increased binding affinity and high degree of nuclease resistance, substantiate the desire to evolve aptamers containing LNA-modified nucleotides to improve aptamers composed of unmodified RNA or DNA. There are two ways to introduce LNA modifications in aptamers. One is to evolve a natural nucleotide aptamer by conventional SELEX processes and then to chemically modify the evolved aptamer sequences with LNA nucleotides (post-SELEX method). Another approach is to use LNA-modified sequence libraries to generate LNA aptamers by normal SELEX-based strategy. So far, the use of LNA in aptamer technology has been limited to the post-SELEX approach. In this direction, Darfeuille *et al*. [59,60] introduced LNA modification to an existing RNA aptamer sequence targeting the TAR RNA element of HIV-1. Surface Plasmon resonance (SPR)–based experiments identified LNA/DNA mixmer oligonucleotides binding to TAR RNA with a dissociation constant in the low nanomolar range comparing favorably to the originally evolved RNA aptamer. Schmidt *et al*. [61] described the capability of LNA modifications to improve the *in vivo* stability of aptamers and their targeting function. Another work showed that an aptamer modified with LNA nucleotides targeting the TAR RNA element of HIV-1 displayed good binding properties and competed with the viral protein Tat for binding to TAR [62]. Further studies from the same lab later reported an improved HIV-1 TAR element binding by modifying a TAR RNA aptamer with LNA/2′-*O*-methyl RNA mixmers [63]. Furthermore, Hernandez *et al*., recently showed the binding affinity of a DNA aptamer selected against avidin can be

significantly improved by introducing just one LNA-G nucleotide. They further demonstrated that 2′-amino LNA-T nucleotide can also be added by maintainging similar $K_D$ values, which will function as a carrier unit for additional molecualr entities [64].

All these results gathered from the post-SELEX approach discussed earlier show the potential of developing LNA aptamers and the need to evolve LNA aptamers by conventional SELEX-based procedures. To apply LNA nucleotides in SELEX selection, their substrate specificities to various DNA or RNA polymerases must be studied. Toward this path, Veedu *et al*., [65–68] and others [69,70] have reported the synthesis and enzymatic incorporation of LNA nucleotides into DNA and RNA oligonucleotides. They successfully applied LNA nucleoside 5′-triphosphates as substrates for polymerases directed primer extension, PCR, and transcription reactions. With these findings, the evolution of LNA aptamers by SELEX processes should be possible in the near future. No LNA-modified aptamer has entered preclinical drug development.

## 9.3. CONCLUDING REMARKS AND FUTURE PROSPECTS

LNA is a highly versatile nucleic acid analogue in the context of nucleic acid base drug development with respect to their remarkable hybridization properties with high affinity and specificity. LNA nucleotides are compatible with other chemistries to make mixmer oligonucleotides with improved hybridization and pharmacokinetic properties for applications in molecular biology research, biotechnology, and RNA targeting. We envision that aptamer technology may prove useful in developing nucleic acid drugs against an array of human health issues. Along this line, we are focusing on evolving LNA aptamers by conventional SELEX processes. The reports discussed in this chapter highlight that LNA will be an important molecule for future development of nucleic acid–based technologies.

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