REVIEW

Locked Nucleic Acids: Promising Nucleic Acid Analogs for Therapeutic Applications

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Locked Nucleic Acid (LNA) is a unique nucleic-acid modification possessing very high binding affinity and excellent specificity toward complementary RNA or DNA oligonucleotides. The remarkable properties exhibited by LNA oligonucleotides have been employed in different nucleic acid-based therapeutic strategies both *in vitro* and *in vivo*. Herein, we highlight the applications of LNA nucleotides for controlling gene expression.

Contents

1. Introduction. – In recent years, nucleic acids-based therapy has attracted significant interest for the treatment of many diseases. It comprises several approaches based on nucleic acid as the active component, which include antisense [1][2], ribozymes [2], short interfering RNA (siRNA) [2–4], microRNA (miRNA) [5][6], and aptamers [7–11]. Nucleic acids composed of naturally occurring DNA or RNA nucleotides pose some limitations which directly affect the versatility because of their poor binding affinity and low degree of nuclease resistance. To overcome these limitations, chemically modified nucleic acids are introduced. A number of nucleic acid analogues have been reported in recent years, among which locked nucleic acid (LNA) modification has proved to be unique and has been used extensively for various applications in chemical biology [12–19].

2. LNA Chemistry and Key Features. – LNA is generally considered to be an RNA mimic in which the ribose sugar moiety is locked by an oxymethylene bridge connecting the C(2′)- and C(4′)-atoms which conformationally restricts LNA monomers (*Fig. 1*) into an *N*-type sugar puckering [12][15][20]. Structural studies by NMR spectroscopy

have shown LNA-containing oligonucleotides to fit into an A-type duplex geometry [21][22].

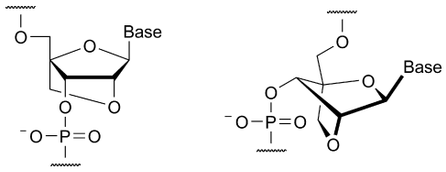

Fig. 1. *Structural representation of LNA monomers*

Several unique properties make LNA a very promising analogue in the field of nucleic acids research. First and most importantly, LNA oligonucleotides possess extremely high binding affinity to complementary DNA and RNA oligonucleotides, as evidenced by thermal denaturation studies, *i.e.*, an increase in melting temperature (T_m) of $+2$ to $+8°$ per LNA monomer compared to the unmodified duplexes [12–16]. In addition, LNAs, as the sequence length can be reduced, also display an improved mismatch discrimination (or improved *Watson–Crick* base-pairing selectivity) relative to unmodified nucleic acids. Furthermore, LNA-modified oligonucleotides show high stability in biological systems (*i.e.*, resistance to enzymatic degradation) [18][19].

3. LNA in Therapeutics and Biotechnology. – Oligonucleotides can modulate gene expression through binding of an antisense oligonucleotide (AON) to a specific messenger RNA (mRNA; *Fig. 2*), pre-mRNA, or non-mRNA by *Watson–Crick* base pairing. Various techniques include classical antisense approach, siRNA, microRNA targeting, DNAzymes *etc.* For effective modulation of gene expression, the usefulness of LNA-modified oligonucleotides has been the concept for many scientific investigations, and successful studies on LNA oligonucleotides for gene-silencing technologies have already been the topic of detailed reviews [23–25].

3.1. *LNA Antisense.* Majority of the antisense experiments conducted with LNA has been focused on mRNA inhibition by RNase H recruitment, although non-RNase H mechanisms have also been reported. *Wahlestedt et al.* 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* [26]. The experiments involved two different LNA sequences, an LNA/DNA mixmer and a LNA/DNA/LNA gapmer targeting the delta opioid receptor (DOR) mRNA in the central nervous system of rats. An efficient knockdown of DOR was induced in both the cases upon direct injection of the oligomers into the rat brain. Thereafter, a number of reports highlighted a broad potential of LNA-modified oligonucleotides in antisense-mediated gene-silencing applications *in vitro* and *in vivo* (for reviews, see [23–25]). A recent report of *Jacobsen et al.*, for example, showed that LNA AONs are effective inhibitors of HIV-1 expression [27].

3.2. *LNA-Modified DNAzyme* (LNAzymes) *for RNA Targeting.* DNAzymes are catalytically active DNA molecules that can function as a specific RNA endonuclease upon binding to a specific RNA sequence [28][29]. In this direction, *Vester et al.* investigated the effect of LNA by incorporating two LNA nucleotides in each of the

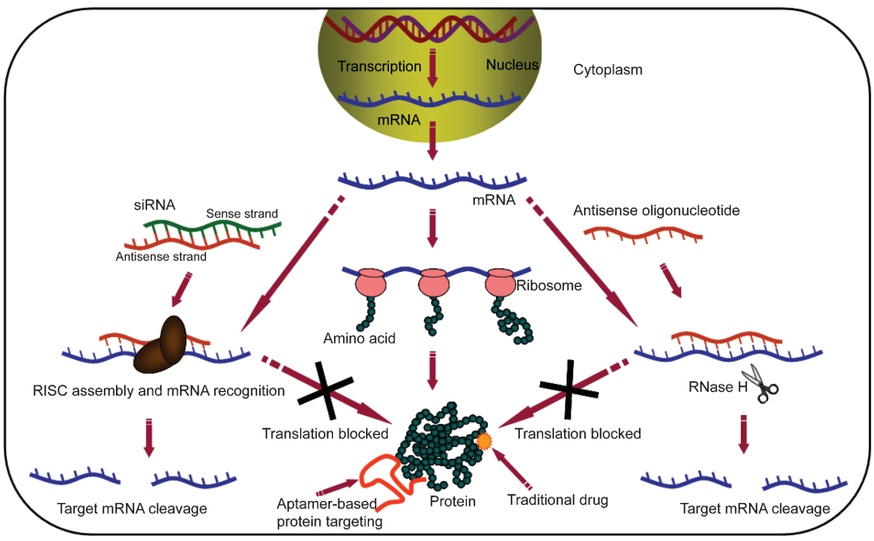

Fig. 2. *Schematic illustration of various nucleic acid-based therapeutic technologies*

binding arms of the DNAzyme yielding an LNAzyme with a highly enhanced efficiency of RNA cleavage [30]. The experiment demonstrated that the cleavage of highly structured targets (a 58n-long RNA with known secondary structure and a 2904n-long 23S ribosomal RNA) was significantly improved using LNAzymes compared to the corresponding unmodified DNAzymes. A similar approach was employed by *Schubert et al.* who incorporated 3–4 LNA monomers at the ends of the binding arms and observed a highly enhanced efficiency of RNA cleavage [31]. 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 hydrolysis by the corresponding unmodified DNAzymes [32]. More recently, *Jacobsen et al.* reported an efficient inhibition of HIV-1 expression by targeting LNAzymes to functionally selected binding sites [27], whereas targeting of miRNAs by using LNAzymes has recently been reported by *Maiti* and co-workers [33].

3.3. *LNA-Modified siRNA* (siLNA). Small interfering RNAs (siRNAs) have emerged as powerful candidates for an efficient knockdown of gene expression in mammalian cells by the RNA-interference (RNAi) pathway [34]. In this approach, siRNA can target complementary mRNA and induce its degradation upon incorporation into the RNA-induced silencing complex (RISC). siRNA themselves are candidates for incorporation of modified nucleotides for improved biostability, and/or effective and selective RNA targeting. In this approach, the application of LNA-modified siRNA, termed siLNA, has been investigated. *Braasch et al.* first conducted a study in mammalian cells using LNA-modified siRNA and observed that the introduction of LNA nucleotides substantially increased the thermal stability of the modified RNA duplex without compromising the efficiency of RNAi [35]. Another

study by *Elmén et al.* showed that systematically modified siRNA duplexes with LNA monomers have remarkably enhanced serum half-life compared to the corresponding unmodified siRNAs [36]. This report also highlighted an improved efficacy of siLNAs on certain RNA motifs relevant for targeting the SARS-CoV virus in addition to provide an evidence that the use of siLNAs reduces sequence related off-target effects. Recently, *Mook et al.* evaluated the effect of LNA-modified siRNA both *in vitro* and *in vivo* [37]. 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 each of *ca.* 10–12 nt in length [38]. In the sisiRNA approach, only the antisense strand is functional, as the nick completely eliminates unintended mRNA targeting by the sense strand. LNA Nucleotides were incorporated to stabilize the sisiRNA constructs, which proved efficient for gene silencing upon transfection into a H1299 lung carcinoma cell line.

3.4. *MicroRNA Targeting Using LNA Probes.* MicroRNAs (miRNAs) constitute a class of short regulatory RNAs (*ca.* 22 nt) that control gene expression post-transcriptionally during development, differentiation, and metabolism [5][6]. Similar to classical AONs 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.* 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 [39]. *Wienholds et al.* 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 [40]. Another report highlighted a sensitive microarray platform using LNA-modified capture probes for miRNA expression profiling [41]. More recently, *Elmén et al.* described effective LNA antimiRs (LNA/DNA mixmer oligonucleotides) with respect to miRNA silencing in non-human primates (target liver-expressed miR-122) [42]. Their findings demonstrate the potential of these compounds as new class of therapeutics for diseases associated with miRNAs.

4. LNA Aptamers. – Aptamers [7–11] 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) [43–46]. The remarkable properties and applications of LNAs highlighted above, particularly their increased binding affinity and high degree of nuclease resistance, substantiate the prospects of using aptamers composed of LNA-modified nucleotides to rival aptamers composed of unmodified RNA or DNA. There are two ways to introduce LNA modifications in aptamers. One is to evolve DNA/RNA aptamers by conventional SELEX processes and then 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 a 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.* introduced LNA modifications to an existing RNA aptamer sequence targeting the trans-activation-responsive (TAR) RNA element of HIV-1 [47][48]. Surface plasmon resonance (SPR) based experiments identified LNA/DNA mixmer oligonucleotides binding to TAR RNA with a dissociation constant in the low nanomolar range. *Schmidt et al.* described the capability of LNA modifications to improve the *in vivo* stability of aptamers and their targeting function [49]. 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 [50]. Further studies from the same laboratory later led to an improved HIV-1 TAR element binding by modifying a TAR RNA aptamer with LNA/2′-*O*-methyl RNA mixmers [51]. All of these results show the potential of developing LNA aptamers and the need to evolve LNA aptamers by conventional SELEX-based procedures. As a first step towards this path, *Veedu et al.* has reported the synthesis and enzymatic incorporation of LNA nucleotides into DNA and RNA oligonucleotides [52–55].

5. Conclusions and Future Prospects. – LNA Oligonucleotides are versatile nucleic-acid analogues with remarkable properties as improved hybridization properties for applications in molecular biology research, biotechnology, and therapeutics. Based on the results discussed above, LNA will likely be an important molecule for future development of nucleic acid-based technologies. We envision that also LNA-based aptamer technology will prove useful in developing nucleic-acid drugs against an array of human diseases.

The *Nucleic Acid Center* is a research center of excellence funded by the *Danish National Research Foundation* for studies on nucleic acid chemical biology. We thank the *Danish National Research Foundation* for financial support.

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Received October 19, 2009