Perspectives on Chemistry and Therapeutic Applications of Locked Nucleic Acid (LNA)

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1. Introduction

1.1. Coverage of Review

This review focuses on the potentials and perspectives of Locked Nucleic Acid (LNA)-based oligonucleotides as therapeutic candidates. It briefly outlines various oligonucleotide-based gene silencing approaches with attention to the practical constraints leading to their downfall, as well as the diverse classes of modified nucleotide analogues that have been widely used until now to modulate gene expression. Their mode of action, characterstics, and inherent drawbacks are also stated.

It provides an exhaustive account of the advances made from the discovery of LNA to the latest developments in the area of LNA research. Beginning from synthetic methods and the different structural analogues of LNA synthesized until now, it gives detailed insight into the multiple novel attributes of LNA that make it preferable over all other classes of modified nucleic acid analogues for *in vivo* applications. An explicit account of the succcessful application of LNA technology in different areas of research such as antisense and antigene strategies, diagnostics, genotyping, and so forth is described along with the current status of LNA in clinical trials. The review concludes with a discussion of the future prospects of LNA technology in the field of therapeutics and genomics.

1.2. Oligonucleotides as Therapeutics

Understanding nature's mute but elegant language of intermolecular communication is the quest of modern biochemical research. Recent advances in molecular biology and biotechnology have enabled us to understand how cryptic and subtle genetic information is converted to a phenotype. The challenge now lies in finding new approaches that will allow the regulation and manipulation of this process in order to develop new pharmaceutical interventions to treat diseases characterized by the aberrant expression or inactivation of certain genes. For a long time now, the immense potential of oligonucleotides as therapeutic tools for the modulation of gene expression has been examined.[1] A modified oligonucleotide, Vitravene (Formivirsen), developed by ISIS Pharmaceuticals, has already been approved for the treatment of cytomegalovirus-induced retinitis,[2] and many other modified oligonucleotides are in phase 1 or phase 2 trials. This reinforces our firm opinion that modified oligonucleotides are excellent candidates for therapeutic applications. Normally, an agent designed to alter gene expression can act either at the level of DNA to inhibit transcription (**the antigene approach**) or at the level of RNA to inhibit translation (**the antisense approach**). Most agents silence gene expression by using the antisense strategy, wherein stable base pairing of an antisense oligonucleotide to the target mRNA blocks the translation apparatus or cleaves the target mRNA by recruitment of RNase H.[1] Steric blockade

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of the translation is achieved when an antisense oligonucleotide is directed to the 5′-terminus of the target, thus, blocking the binding and assembly of the translational machinery, while RNase H-mediated cleavage occurs when the antisense oligonucleotide binds to the target strand at any site to produce a duplex conformation of DNA•RNA heteroduplex. This conformation serves as a recognition element of RNase H recruitment, leading to the cleavage of the RNA strand of the heteroduplex. RNA blockage by small interfering RNA (siRNA) can also inhibit translation, thereby greatly altering the level of gene expression. siRNAs result when transposons, viruses, or endogenous genes express long double-stranded RNA (dsRNA), or when dsRNA is introduced experimentally into plant or animal cells to trigger gene silencing, a process known as RNA interference (RNAi) (Figure 1).[1] Of all the silencing modes listed, the degradation of mRNA by RNase H and siRNA is considered to be extremely potent in terms of ensuing complete mRNA breakdown.

In theory, use of oligonucleotide-based antisense and antigene strategies seems quite feasible where high-affinity

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hybridization between the oligonucleotide and the target strand serve to alter gene expression. But in practice, use of these strategies has fallen from favor owing to difficulties in predicting: (i) the accessible sites or structures of the target against which the oligonucleotide must be directed, (ii) the optimum dose of the agent which will avoid toxic effects, and (iii) the specificity and *in vivo* stability of the oligonucleotide.

1.3. Overview of Different Modified Nucleotide Analogues

During the last couple of decades, exploration and examination of novel structurally modified oligonucleotides that act as potent and selective therapeutic agents has gained momentum and has led to the development of analogues which have the desired properties and minimum toxicity. In general, there exist three types of modified nucleotides, namely, analogues with unnatural bases, analogues with modified sugars (especially the 2′-position), and those with an altered phosphodiester backbone (Figure 2). Among the most widely used modified oligonucleotide analogues are **Phosphorothioates** (PS) (Figure 2), which represent an important class of first generation antisense (AS) molecules. The operative modification in these analogues involves substitution of one of the nonbridging oxygens in the phosphodiester linkage with a sulfur atom. This produces a phosphorothioate linkage (PS) that destabilizes the base pairing somewhat but renders the AS molecule resistant to nuclease digestion. Although the presence of a polyanionic backbone allows these analogues to interact with proteins and thereby increase their half-life in biological fluids,[3] this seemingly advantageous property also contributes significantly to cellular toxicity.[4] The second generation of therapeutic molecules is mainly comprised of nucleotides with an alkyl modification at the 2′-position of the ribose,

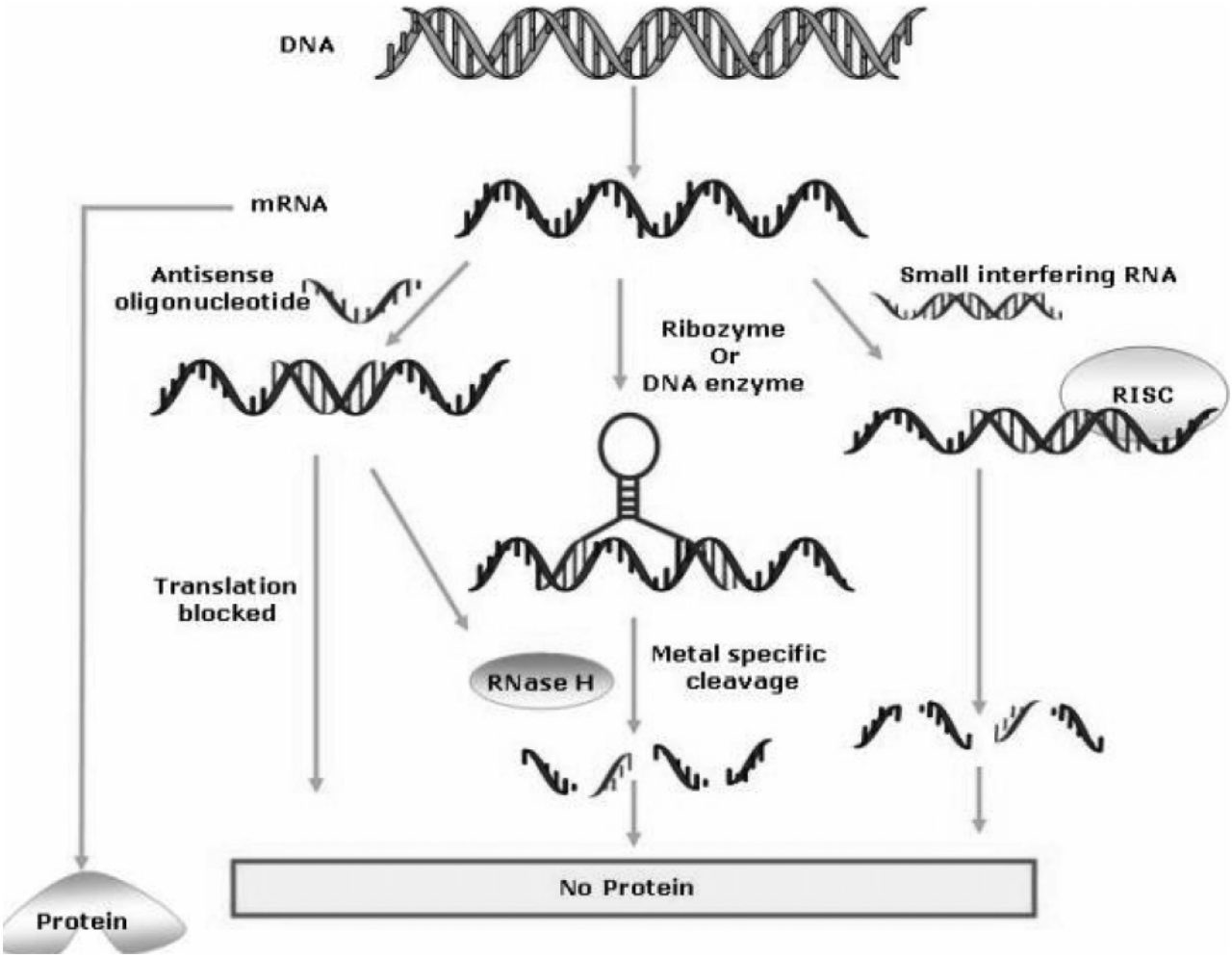


Figure 1. Conceptual representation of antisense-mediated gene-silencing approaches. Antisense oligonucleotides may block translation of mRNA or induce RNase H-mediated degradation of their targets. Cleavage of mRNA also results from the catalytic activity of the DNAzymes or ribozymes. Alternatively, small interfering RNA (siRNA), a class of short 21−23 nt RNA duplex molecules, might stimulate the cellular machinery (RISC) to cleave other single-stranded RNA having the sequence complementary to siRNA.

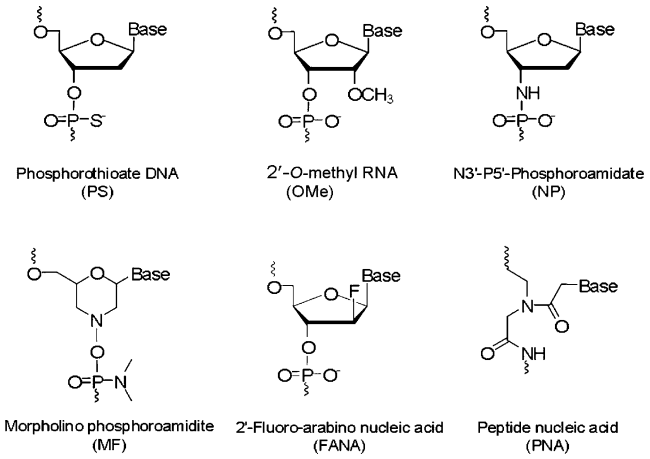


Figure 2. Examples of synthetic nucleic acid analogues containing backbone modifications.

for example, **2′-*O*-methyl** and **2′-*O*-methoxy ethyl** RNA. These are less toxic than PS oligodeoxynucleotides, possess a higher binding affinity to their respective target sequence, and can effectively mediate antisense effects by steric block hindrance of translation, but they lack the ability to activate RNase H.[5,6]

The RNase H-independent mechanism of action of such modified oligonucleotides can be exploited to alter mRNA splicing, in which blocking a given splice site leads to an increased expression of an alternatively spliced variant.[7] The third generation of modified oligonucleotides includes morpholino oligonucleotides, N3′-P5′-phosphoroamides, 2′-deoxy, 2′-flouro-β-D-arabino (FANA) nucleic acid, and peptide nucleic acids. **N3′-P5′-phosphoroamidates** (NPs) have a modified phosphate backbone where the 3′-hydroxyl group of the 2′-deoxyribose ring is replaced with a 3′-amino group. NPs exhibit high affinity and high nuclease resistance toward complementary RNA strand.[8] Similar to 2′-*O*-methyl RNA, NPs also achieve their antisense effect by steric blockade of translation. However, both the analogues can be made RNase H active with the use of the so-called gapmer technology, in which a central core of DNA or phosphorothioate DNA monomers flanked by modified nucleotides at each end induces RNase H recruitment. The potency of NPs as AS has already been demonstrated *in vivo*, where they proved to be superior to PS in specifically down-regulating the expression of the *c-myc* gene.[9] Because NPs do not induce RNase H cleavage of the target RNA, they might prove useful where RNA integrity needs to be maintained, such as in the case of splicing modulation. **Morpholino oligonucleotides** (MF), which are examples of nonionic DNA analogues, have ribose replaced by a morpholino moiety and contain phosphoroamidate inter-subunit linkages instead of phosphodiester bonds. Most of the work on

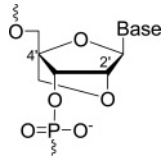


Figure 3. Locked nucleic acid (LNA).

morpholino compounds has focused on gene regulation during the development of zebrafish.[10] MFs do not activate RNase H but can be targeted toward the 5′ untranslated region or the first 25 bases downstream of the start codon to block translation by preventing ribosome-binding. Because their backbone is uncharged, MFs are unlikely to have unwanted interactions with nucleic acid binding proteins. The target affinity of MFs is similar to that of isosequential DNA oligonucleotides, but is lower than that of many of the other modifications described here. Another important compound is **2′-deoxy-2′-fluoro-*β*-D-arabino nucleic acid analogue** (FANA), a 2′ epimer of RNA and the first uniformly sugar-modified AS oligonucleotide that is reported to induce RNase H cleavage of a bound RNA molecule.[11] The **Peptide Nucleic Acids** (PNAs)[12,13] are another class of extensively studied modified DNA analogues. They consist of a synthetic peptide backbone formed from *N*-(2-aminoethyl)-glycine units, which results in achiral and uncharged molecules. Since these PNAs are uncharged compounds, their consequent poor water solubility relative to DNA is a major disadvantage compared to other modified DNA analogues. Nevertheless, PNAs can bind to DNA as well as RNA to form sequence-specific hydrogen-bonded structures that are chemically stable, resistant to enzymatic cleavage, and exhibit higher thermal stability. PNA oligomers recognize duplex homopurine DNA sequences to which they bind by a strand invasion mechanism[12] to form a stable PNA−DNA−PNA triplex. Thus, they can exhibit an antigene effect by arresting transcription and an antisense effect by steric blockade of RNA processing enzymes. However, a successful therapeutic application of PNAs still awaits the development of efficient methods for its uptake and delivery into cells.

One of the most promising candidates of chemically modified nucleotides developed in the past few years is the **Locked Nucleic Acid** (LNA). LNA bases are ribonucleotide analogues containing a methylene linkage between the 2′-oxygen and 4′-carbon of the ribose ring (Figure 3). The constraint on the sugar moiety results in a locked 3′-endo conformation that prepares the base for high affinity hybridization.[14−21] Its close structural resemblance to RNA, high affinity and specificity toward the target strand, high *in vivo* stability, lack of toxicity, and ease of transfection into cells have contributed to its success as a promising tool in therapeutics and functional genomics. Furthermore, LNA oligonucleotides can be synthesized using conventional phosphoramidite chemistry, thus, allowing automated synthesis of fully modified LNA and chimeric oligonucleotides such as LNA/DNA and LNA/RNA.[15,18] Finally, their charged phosphate backbone allows ready delivery into cells using standard cationic transfection agents.[21]

2. LNA: An Insight

2.1. Family

A number of structural analogues of LNA have been synthesized (Figure 4**)** and investigated for various attributes.[22,23] Although most members of the LNA family exhibit high binding affinity to RNA, the binding efficiency of *β*-D-LNA (parent LNA) is known to be the highest of all the diastereomeric forms. Among the other stereoisomers, α-L-LNA caught the attention as it displayed thermostability properties only slightly inferior to the parent LNA. Such remarkable binding affinity and specificity obtained for LNA and α-L-LNA, when fully and partially modified, have established these molecules as unique nucleic acid mimics.[22] These molecules, along with other derivatives containing the 2′-heteroatom to 4′-C linkage (Figure 4), were tested *in vivo* for their antisense efficacy and were found to be very effective.[23] However, each of these molecules exhibit different pharmacokinetic profiles, thereby, opening new

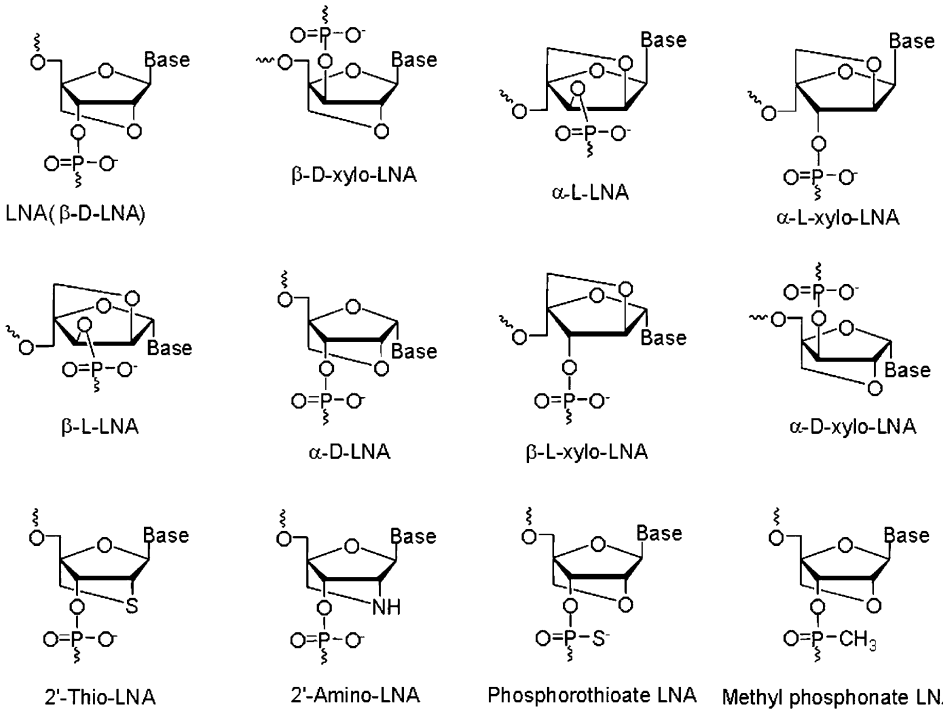


Figure 4. LNA (represented by *β*-D-LNA) and its molecular family.

avenues towards the choice of LNA chemistry to suit the desired profiles.

2.2. Synthesis

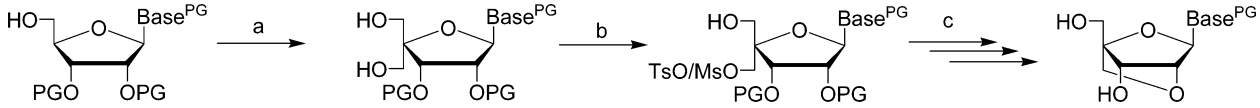
Two strategies have been used to synthesize LNA monomers: a linear strategy using nucleosides as the starting material,[24−27] and a convergent strategy wherein an appropriately modified glycosyl donor is synthesized and then coupled with the nucleobase to give the modified nucleoside.[14,15,18,27−33] Following the linear approach, LNA-U[24−26] and LNA-A[27] nucleosides have been synthesized with uridine and adenosine as starting materials. Despite some advantages, such as the relatively short series of chemical transformations required and the cheap RNA nucleoside starting materials, the linear strategy has its limitations. Two key reactions in the synthetic pathway, that is, introduction of the additional hydroxymethyl group at the 4′-C-position of the protected RNA nucleoside (Scheme 1: step a) and the regioselective tosylation (or mesylation) of the 4′-*C*-hydroxymethyl group (Scheme 1: step b), generally produce rather small yields.

Alternatively, the LNA nucleosides containing all the natural nucleobases have been synthesized using the convergent strategy.[15] All of the chemical reactions involved in the optimized synthesis of LNA containing thymine,[31,32] 4-*N*-acetyl and 4-*N*-benzoyl cytosine,[31] 6-*N*-benzoyl adenine,[31] and 2-*N*-isobutyryl guanine[31] as nucleobases were subsequently refined and published (Scheme 2).

In the convergent synthesis, the key intermediate **2** was prepared in a three-step protocol from 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose **1**.[34] The starting sugar **1** is commercially available but can also be synthesized from its gluco-epimer via oxidation and selective reduction in 58% yield with a one-pot, two-step procedure using DMSO/Ac_2O/$NaBH_4$, or in 73% yield with a two-pot, two-step procedure using DMSO/P_2O_5/$NaBH_4$. The 5,6-isopropylidene protecting group in **1** was selectively removed using 80% aqueous acetic acid, the 5,6-glycol was oxidatively cleaved by periodate, and the resulting 5-aldehyde derivative was reacted with formaldehyde. This was followed by an *in situ* crossed Cannizzaro reaction with excess formaldehyde, which yielded the desired 4-*C*-hydroxymethyl derivative **2**. The diol **2** was permesylated, and subsequent acetolysis followed by acetylation gave the anomeric mixture **3** in 67% yield (from **1**).[32] This mixture was then used as a common glycosyl donor in the coupling reactions with different nucleobases. The protected nucleobases were stereoselectively coupled with **3** under Vorbrüggen's conditions[36] to give the corresponding 4′-*C*-nucleosides. Subsequently, a one-pot reaction sequence consisting of 2′-O-deacetylation followed by intramolecular cyclization under alkaline conditions gave the protected LNA nucleosides **5a**−**d**. Additionally, due to the lability of the 4-*N*-acetyl group of nucleoside **4b** and 6-*N*-benzoyl group of nucleoside **4c**, the reaction was allowed to proceed until complete nucleobase deprotection was accomplished, furnishing nucleosides **5b** and **5c**, respectively. The 5′-*O*-mesyl groups in the nucleosides **5a**−**d** were displaced by nucleophilic substitution using sodium benzoate. Subsequent saponification of the 5′-benzoates and catalytic removal of the 3′-*O*-benzyl groups (and protection of the exocyclic amino groups of LNA-A and LNA-C) afforded the free LNA diols **7a**−**d**. Formic acid was used as the hydrogen donor for debenzylation of **6d** to prevent the removal of the base-labile 2-*N*-isobutyryl group. LNA diols were 5′-O-dimethoxytritylated[14] and subsequently quantitatively converted into their phosphoramidites derivatives by reaction with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite and using 4,5-dicyanoimidazole as an activator suitable for automated incorporation into oligonucleotides.[37]

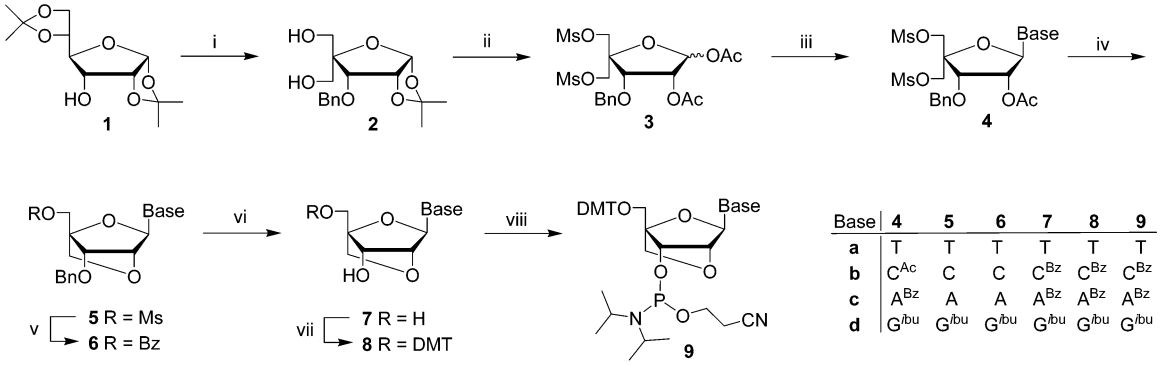
In another approach, the phosphoramidites of LNA-T and LNA-U have been transformed into their 4-triazolo deriva-

Scheme 1. Linear Approach: General Scheme for the Synthesis of LNA Nucleosides[a]



[a] Base, nucleobase; PG, protecting group; Ms, methanesulphonyl; Ts, *p*-toluenesulphonyl.

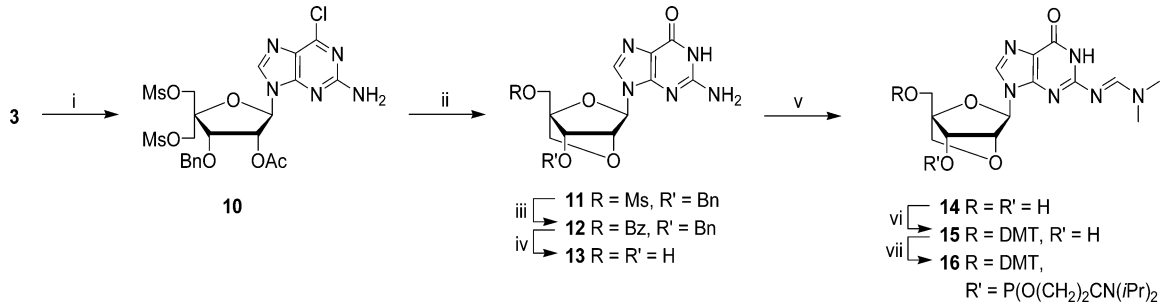
Scheme 2. Convergent Synthesis of LNA Phosphoramidites[31,32] [a]



Base	4	5	6	7	8	9
a	T	T	T	T	T	T
b	C^{Ac}	C	C	C^{Bz}	C^{Bz}	C^{Bz}
c	A^{Bz}	A	A	A^{Bz}	A^{Bz}	A^{Bz}
d	G^{ibu}	G^{ibu}	G^{ibu}	G^{ibu}	G^{ibu}	G^{ibu}

[a] Reagents (and yields): (i) (a) BnBr, NaH, THF; (b) 80% aq. AcOH; (c) $NaIO_4$, THF, H_2O; (d) HCHO, aq. NaOH, dioxane; (ii) (a) MsCl, pyridine, CH_2Cl_2; (b) Ac_2O, AcOH, concd H_2SO_4 (67% from **1**);[32] (iii) nucleobase, *N*,*O*-bis(trimethylsilyl)acetamide, TMS-triflate, CH_3CN or 1,2-dichloroethane (**4a**, 88%; **4b**, 82%; **4c**, 68%; **4d**, 84%); (iv) aq. NaOH, THF or dioxane (+NH_4OH for **5c**) (**5a**, 94%; **5b**, 87%; **5c**, 78%; **5d**, 85%); (v) NaOBz, DMF (**6a**, 86%; **6b**, 93%; **6c**, 84%; **6d**, 92%); (vi) (a) aq. NaOH, THF; (b) 20% $Pd(OH)_2$/C, HCO_2NH_4, MeOH (**7a**, 76%) or (a) 20% $Pd(OH)_2$/C, HCO_2NH_4, MeOH; (b) NH_4OH; (c) Bz_2O, pyridine; (d) aq. NaOH, EtOH (**7b**, 72%) or (a) 20% $Pd(OH)_2$/C, HCO_2NH_4, MeOH, dioxane (b) BzCl, pyridine; (c) aq. NaOH, pyridine, EtOH (**7c**, 75%) or (a) aq. NaOH, EtOH, pyridine; (b) 10% Pd/C, HCO_2H, MeOH (**7d**, 70%); (vii) DMTCL, pyridine (**8a**, 93%; **8b**, 68%; **8d**, 86%);[15] (viii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, 4,5-dicyanoimidazole (1 M solution in CH_3CN), CH_2Cl_2 (≥95%);[37] T = thymin-1-yl, C^{Ac} = 4-*N*-acetylcytosin-1-yl, C^{Bz} = 4-*N*-benzoylcytosin-1-yl, A^{Bz} = 6-*N*-benzoyladenin-9-yl, G^{ibu} = 2-*N*-isobutyrylguanin-9-yl, Bn = benzyl, Ms = methanesulphonyl, DMT = 4,4′-dimethoxytrityl.

Scheme 3. Efficient Synthesis of LNA-G Phosphoramidite by Regiospecific Vorbrüggen Coupling of 2-Amino-6-chloropurine to the Carbohydrate Moiety[a]



[a] Reagents (and yields): (i) 2-amino-6-chloropurine, *N*,*O*-bis(trimethylsilyl)acetamide, TMS-triflate, 1,2-dichloroethane (90%); (ii) $HOCH_2CH_2CN$, NaH, THF (86%); (iii) NaOBz, DMSO (95%); (iv) (a) MsOH, CH_2Cl_2; (b) Amberlyst A-26 (OH^- (aq), EtOH); (c) aq. NaOH, EtOH; (v) (a) $(CH_3O)_2CHN(CH_3)_2$, DMF; (vi) 4,4′-dimethoxytrityl chloride, pyridine (84% from **12**); (vii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, 4,5-dicyanoimidazole (1 M solution in CH_3CN), CH_2Cl_2, DMF (88%).

tives, which were then easily converted into the cytidine derivatives (LNA-^{5Me}C and LNA-C, respectively) by treatment with concentrated ammonia after oligonucleotide synthesis.[20,26]

LNA and LNA−DNA chimeras containing phosphodiester or phosphorothioate linkages, or a mixture thereof, can be assembled by standard DNA synthesizers. Compared to the standard protocols used for DNA synthesis, slightly longer coupling and oxidation times are needed for efficient oligomerization of the LNA phosphoramidites.[38]

During the synthesis of LNA-G (Scheme 3), the glycosylation reaction applied to 2-*N*-isobutyryl guanine furnished an isomeric mixture of the N-9/N-7 nucleosides in a ratio of about 9:1,[31] and chromatographic purification was required after the ring closing reaction to obtain an isomerically pure compound **5d**. The strategy devised to overcome this problem (Scheme 3)[31] involved the glycosylation reaction of 2-amino-6-chloropurine with the glycosyl donor **3** to form only the N-9 regioisomer selectively. Reaction of nucleoside **10** with 3-hydroxypropionitrile in the presence of sodium hydride resulted in the ring closure to form the bicyclic LNA skeleton with concomitant transformation of the 2-amino-6-chloropurine nucleobase into a guanine nucleobase, that is, nucleoside **11**. Mild removal of the 5′-*O*-mesyl group from **11** by the standard two-step procedure and 3′-O-debenzylation with methane sulfonic acid furnished the fully deprotected LNA guanine **13**. The guanine nucleobase was protected with the dimethylformamidine protective group, which is cleaved rapidly using the standard oligonucleotide deprotection protocol. Standard 5′-O-dimethoxytritylation followed by 3′-O-phosphitylation furnished the LNA-G phosphoramidite **16**.

Following the convergent synthetic strategy principle, analogous synthetic procedures were used for the synthesis of second generation LNA purine nucleotides containing hypoxanthine (LNA-I),[39] 2,6-diaminopurine (LNA-D),[33,39] and 2-aminopurine (LNA-2AP)[39] as nucleobases. All of the three nucleotides hybridized very efficiently to complementary DNA following the Watson−Crick base pairing rules with increased binding affinity when compared to the isosequential DNA oligonucleotides. Replacement of an internal LNA-A monomer by an LNA-D monomer further stabilized the duplex by the potential formation of three hydrogen bonds with DNA (T). LNA-2AP monomer, when incorporated into an LNA strand, also showed a strong preference for hybridization with a DNA-T nucleotide, which suggests the formation of stable bidentate hydrogen bonding in LNA−DNA duplexes. The LNA-I monomer, which contains hyoxanthine as a nucleobase, behaved similarly to the LNA-G nucleotide in that it preferred binding to the DNA-C nucleotide.

2.3. Attributes

2.3.1. Hybridization

The potential for LNA to act as a therapeutic tool lies in its ability to mediate high-affinity hybridization with the complementary RNA, as well as with ssDNA, without the loss of sequence specificity. In fact, LNAs exhibit mismatch discrimination equal or superior to native DNA. The hybridization properties of LNA-containing oligonucleotides have been evaluated in different sequence contexts with oligomers ranging from 6 to 20 nucleotides with varying levels of LNA content including, for example, fully modified LNA, LNA/DNA mixmers, LNA/RNA mixmers, and LNA/PS-DNA mixmers.[15,18,40,42] The unprecedented hybridization potential of LNA with either RNA or DNA targets is reflected in the increased thermostability of the LNA-containing duplexes. Substitution by an LNA monomer leads to an increase of T_m values up to +1 to +8 °C against DNA and an increase of +2 to +10 °C against RNA.[5,18,21,44] This is possibly the largest increase in thermostability observed for a nucleic acid analogue, but it does saturate when the relative substitution by LNA monomer reaches to about 50% of the total residues in the LNA/DNA chimera.[15,21,41] Furthermore, the impact on the thermostability depends on the oligomer length and composition. Since, LNA•LNA base pairing is very strong, the self-annealing capacity of LNA must be taken into account when designing fully modified LNA or LNA mixmers with a large number of LNA substitutions.

2.3.2. Thermodynamics

Accurate predictions of nucleic acid hybridization thermodynamics are fundamental to primer and probe design. The novelty and versatility of LNA as an oligonucleotide-based therapeutic agent has led to its multifarious applications in genomics and diagnostics. However, the much needed insight into its hybridization thermodynamics, which is required for accurate primer and probe design, was still lacking. One of the recent reports[45] characterizing the thermodynamics of LNA-based oligonucleotides used simple UV-melting studies to explore the sequence dependence of thermodynamic parameters for LNA−DNA duplex formation. Table 1 presents the data obtained by melting 100 LNA-

Table 1. Experimental Thermodynamic Values for LNA-Containing DNA Oligonucleotides[a]

LNA-substituted DNA oligonucleotides			results from absorbance melting curves				changes due to LNA substitution			
name	sequence (5′ to 3′)	length, trinuc	$\Delta H°$ (kcal/ mol)	$\Delta S°$ (e.u.)	$\Delta G°_{37}$ (kcal/mol)	T_m (°C)	ΔT_m (°C)	$\Delta\Delta H°$ (kcal/mol)	$\Delta\Delta S°$ (e.u.)	$\Delta\Delta G°_{37}$ (kcal/mol)
D1	GTCGAACAGC	10	−80.6 ± 1.6	−221 ± 5	−12.1 ± 0.1	51.8				
L1a	GTC**G**^{L}AACAGC	C**G**^{L}A	−74.2 ± 2.8	−202 ± 9	−11.6 ± 0.1	51.2	−0.6	6.5 ± 3.2	19.4 ± 9.9	0.43 ± 0.16
L1b	GTCG**A**^{L}ACAGC	G**A**^{L}A	−76.2 ± 1.5	−206 ± 5	−12.3 ± 0.1	53.8	2.0	4.4 ± 2.2	14.9 ± 6.8	−0.24 ± 0.12
L1c	GTCGA**A**^{L}CAGC	A**A**^{L}C	−77.2 ± 1.3	−209 ± 4	−12.4 ± 0.1	53.9	2.1	3.4 ± 2.1	12.0 ± 6.4	−0.32 ± 0.11
L1d	GTCGAA**C**^{L}AGC	A**C**^{L}A	−75.5 ± 1.1	−202 ± 3	−12.8 ± 0.1	56.1	4.3	5.1 ± 2.0	18.7 ± 6.0	−0.71 ± 0.11
D2	ATCTATCCGGC	11	−80.3 ± 1.0	−219 ± 3	−12.3 ± 0.1	52.7				
L2a	ATC**T**^{L}ATCCGGC	C**T**^{L}A	−79.3 ± 2.6	−213 ± 8	−13.3 ± 0.2	57.3	4.6	1.0 ± 2.7	6.5 ± 8.3	−1.01 ± 0.18
L2b	ATCTA**T**^{L}CCGGC	T**A**^{L}T	−76.8 ± 2.0	−207 ± 6	−12.5 ± 0.1	54.5	1.8	3.5 ± 2.2	12.2 ± 6.7	−0.23 ± 0.13
L2c	ATCTA**T**^{L}CCGGC	A**T**^{L}C	−73.9 ± 2.4	−198 ± 7	−12.5 ± 0.2	55.4	2.7	6.4 ± 2.6	21.5 ± 7.9	−0.27 ± 0.16
L2d	ATCTAT**C**^{L}CGGC	T**C**^{L}C	−74.0 ± 1.3	−198 ± 4	−12.7 ± 0.1	56.1	3.5	6.3 ± 1.6	21.9 ± 4.9	−0.44 ± 0.10
L2e	ATCTATC**C**^{L}GGC	C**C**^{L}G	−76.6 ± 1.0	−206 ± 3	−12.8 ± 0.1	55.9	3.2	3.7 ± 1.4	13.6 ± 4.3	−0.53 ± 0.08
D3	CGCTGTTACGC	11	−79.3 ± 1.2	−214 ± 4	−13.0 ± 0.1	56.1				
L3a	CGC**T**^{L}GTTACGC	C**T**^{L}G	−82.9 ± 1.4	−221 ± 4	−14.2 ± 0.1	60.5	4.3	−3.6 ± 1.8	−7.6 ± 5.5	−1.22 ± 0.13
L3b	CGCT**G**^{L}TTACGC	T**G**^{L}T	−81.0 ± 1.8	−217 ± 5	−13.6 ± 0.1	58.4	2.3	−1.7 ± 2.1	−3.4 ± 6.3	−0.63 ± 0.15
L3c	CGCTG**T**^{L}TACGC	G**T**^{L}T	−81.1 ± 1.9	−217 ± 6	−13.8 ± 0.1	59.2	3.1	−1.8 ± 2.2	−3.2 ± 6.7	−0.81 ± 0.16
L3d	CGCTGTT^{L}ACGC	TT^{L}A	−78.2 ± 2.4	−208 ± 7	−13.6 ± 0.2	59.2	3.1	1.0 ± 2.7	5.3 ± 8.0	−0.62 ± 0.19
L3e	CGCTGTT**A**^{L}CGC	TA^{L}C	−78.7 ± 1.4	−211 ± 4	−13.3 ± 0.1	57.6	1.5	0.6 ± 1.8	2.8 ± 5.6	−0.30 ± 0.12
D4	GGACCTCGAC	10	−77.2 ± 1.2	−209 ± 4	−12.3 ± 0.1	53.6				
L4a	GGA**C**^{L}CTCGAC	A**C**^{L}C	−74.5 ± 1.4	−198 ± 4	−13.3 ± 0.1	58.7	5.1	2.7 ± 1.8	11.6 ± 5.6	−0.94 ± 0.12
L4b	GGAC**C**^{L}TCGAC	C**C**^{L}T	−74.1 ± 1.6	−197 ± 5	−13.0 ± 0.1	57.9	4.2	3.1 ± 2.0	12.4 ± 6.0	−0.74 ± 0.13
L4c	GGACC**T**^{L}CGAC	C**T**^{L}C	−74.7 ± 1.1	−200 ± 3	−12.6 ± 0.1	55.5	1.9	2.4 ± 1.7	8.8 ± 5.0	−0.29 ± 0.10
L4d	GGACCT**C**^{L}GAC	T**C**^{L}G	−75.0 ± 1.4	−200 ± 4	−13.1 ± 0.1	57.8	4.2	2.2 ± 1.9	9.6 ± 5.6	−0.78 ± 0.12
D5	CCATTGCTACC	11	−82.4 ± 1.1	−227 ± 3	−11.9 ± 0.1	50.9				
L5a1	CC**A**^{L}TTGCTACC	C**A**^{L}T	−75.8 ± 1.6	−207 ± 5	−11.6 ± 0.1	50.6	−0.3	6.6 ± 1.9	20.1 ± 5.9	0.35 ± 0.09
L5a	CCA**T**^{L}TGCTACC	A**T**^{L}T	−77.2 ± 1.9	−209 ± 6	−12.3 ± 0.1	53.7	2.8	5.2 ± 2.1	18.0 ± 6.5	−0.42 ± 0.12
L5b	CCATT^{L}GCTACC	TT^{L}G	−78.6 ± 1.5	−213 ± 5	−12.6 ± 0.1	54.4	3.5	3.8 ± 1.8	14.2 ± 5.6	−0.64 ± 0.10
L5c	CCATT**G**^{L}CTACC	T**G**^{L}C	−77.3 ± 1.1	−210 ± 3	−12.1 ± 0.1	52.5	1.6	5.1 ± 1.5	16.9 ± 4.7	−0.15 ± 0.07
L5d	CCATTG**C**^{L}TACC	G**C**^{L}T	−79.4 ± 1.1	−214 ± 4	−13.0 ± 0.1	56.0	5.1	3.0 ± 1.6	13.0 ± 4.8	−1.04 ± 0.08
D6	GGTGCCAA	8	−51.6 ± 0.9	−138 ± 3	−8.65 ± 0.02	38.7				
L6a	GG**T**^{L}GCCAA	G**T**^{L}G	−52.3 ± 1.6	−137 ± 5	−9.7 ± 0.1	44.8	6.2	−0.7 ± 1.8	1.0 ± 5.6	−1.01 ± 0.06
L6b	GGT**G**^{L}CCAA	T**G**^{L}C	−50.1 ± 1.4	−132 ± 4	−9.31 ± 0.04	42.9	4.2	1.5 ± 1.6	6.8 ± 5.2	−0.66 ± 0.04
L6b2	GGTG**C**^{L}CAA	G**C**^{L}C	−52.5 ± 2.0	−138 ± 6	−9.8 ± 0.1	45.9	7.3	−0.9 ± 2.2	0.9 ± 6.8	−1.19 ± 0.07
L6c	GGTGC**C**^{L}AA	C**C**^{L}A	−49.6 ± 1.5	−130 ± 5	−9.44 ± 0.04	43.8	5.1	2.0 ± 1.7	8.9 ± 5.4	−0.78 ± 0.05
D7	GGAACAAGATGC	12	−90.8 ± 1.0	−250 ± 3	−13.3 ± 0.1	54.9				
L7a	GGAAC**A**^{L}AGATGC	C**A**^{L}A	−88.5 ± 1.3	−242 ± 4	−13.4 ± 0.1	55.8	0.9	2.3 ± 1.6	7.7 ± 4.9	−0.11 ± 0.10
L7b	GGAACAA^{L}GATGC	A**A**^{L}G	−89.3 ± 2.2	−243 ± 7	−13.9 ± 0.2	57.6	2.6	1.5 ± 2.4	6.6 ± 7.2	−0.59 ± 0.16
L7c	GGAACAA**G**^{L}ATGC	A**G**^{L}A	−89.4 ± 1.7	−242 ± 5	−14.2 ± 0.1	58.6	3.7	1.4 ± 1.9	7.3 ± 5.8	−0.87 ± 0.13
L7d	GGAACAAG**A**^{L}TGC	G**A**^{L}T	−89.8 ± 1.1	−244 ± 3	−14.0 ± 0.1	57.6	2.7	1.0 ± 1.4	5.2 ± 4.4	−0.64 ± 0.09
D8	CACGGCTC	8	−58.5 ± 1.3	−158 ± 4	−9.7 ± 0.1	44.0				
L8a1	CA**C**^{L}GGCTC	A**C**^{L}G	−66.5 ± 3.6	−179 ± 11	−11.0 ± 0.2	49.8	5.7	−8.0 ± 3.8	−21 ± 12	−1.33 ± 0.18
L8a	CAC**G**^{L}GCTC	C**G**^{L}G	−59.5 ± 1.6	−159 ± 5	−10.3 ± 0.1	47.4	3.4	−1.0 ± 2.0	−1.2 ± 6.3	−0.63 ± 0.10
L8b	CACG**G**^{L}CTC	G**G**^{L}C	−58.9 ± 2.2	−155 ± 7	−10.7 ± 0.1	49.9	5.9	−0.4 ± 2.6	2.3 ± 7.9	−1.06 ± 0.13
L8c	CACGG**C**^{L}TC	G**C**^{L}T	−64.4 ± 3.4	−172 ± 11	−11.2 ± 0.2	51.2	7.2	−5.9 ± 3.7	−14 ± 11	−1.53 ± 0.18
D9	GCAGGTCTGC	10	−70.5 ± 1.3	−188 ± 4	−12.1 ± 0.1	54.6				
L9a	GC**A**^{L}GGTCTGC	C**A**^{L}G	−71.9 ± 1.8	−190 ± 5	−12.9 ± 0.1	57.7	3.2	−1.4 ± 2.3	−2.2 ± 7.0	−0.81 ± 0.15
L9b	GCA**G**^{L}GTCTGC	A**G**^{L}G	−76.5 ± 1.4	−202 ± 4	−14.0 ± 0.1	61.5	6.9	−6.1 ± 1.9	−13.7 ± 5.9	−1.90 ± 0.14
L9c	GCAG**G**^{L}TCTGC	G**G**^{L}T	−70.7 ± 2.2	−186 ± 7	−13.0 ± 0.2	58.4	3.8	−0.2 ± 2.6	1.8 ± 7.9	−0.86 ± 0.18
D10	GTAGCGATGTA	11	−75.3 ± 1.2	−205 ± 4	−11.8 ± 0.1	51.9				
L10a	GTA**G**^{L}CGATGTA	A**G**^{L}C	−77.4 ± 1.4	−210 ± 4	−12.4 ± 0.1	53.9	2.0	−2.1 ± 1.8	−4.9 ± 5.6	−0.54 ± 0.10
L10b	GTAG**C**^{L}GATGTA	G**C**^{L}G	−72.8 ± 2.4	−194 ± 7	−12.7 ± 0.2	56.6	4.7	2.6 ± 2.7	11.2 ± 8.2	−0.87 ± 0.17
L10c	GTAGC**G**^{L}ATGTA	C**G**^{L}A	−72.3 ± 1.8	−194 ± 6	−12.0 ± 0.1	53.6	1.7	3.0 ± 2.2	10.4 ± 6.6	−0.21 ± 0.12
L10d	GTAGCGA**T**^{L}GTA	A**T**^{L}G	−73.7 ± 2.8	−196 ± 8	−13.0 ± 0.2	57.7	5.8	1.6 ± 3.0	9.0 ± 9.1	−1.16 ± 0.20
D11	ACGTCTTCG	9	−57.2 ± 1.6	−154 ± 5	−9.35 ± 0.04	42.4				
L11a1	AC**G**^{L}TCTTCG	C**G**^{L}T	−57.4 ± 1.6	−154 ± 5	−9.46 ± 0.04	43.0	0.6	−0.1 ± 2.3	−0.1 ± 7.1	−0.11 ± 0.06
L11a	ACG**T**^{L}CTTCG	G**T**^{L}C	−58.1 ± 2.0	−155 ± 6	−10.2 ± 0.1	47.0	4.7	−0.9 ± 2.5	−0.2 ± 7.9	−0.85 ± 0.08
L11b	ACGT**C**^{L}TTCG	T**C**^{L}T	−63.0 ± 1.4	−168 ± 4	−10.8 ± 0.1	49.6	7.2	−5.7 ± 2.2	−13.7 ± 6.7	−1.49 ± 0.08
L11c	ACGTC**T**^{L}TCG	C**T**^{L}T	−59.1 ± 2.1	−158 ± 7	−10.0 ± 0.1	45.7	3.3	−1.9 ± 2.7	−4.0 ± 8.3	−0.64 ± 0.08
L11e	ACGTCTT^{L}CG	TT^{L}C	−58.9 ± 1.8	−158 ± 6	−10.0 ± 0.1	45.5	3.1	−1.7 ± 2.4	−3.4 ± 7.6	−0.60 ± 0.07
D13	TTGGGAGTAGC	11	−72.6 ± 1.3	−197 ± 4	−11.5 ± 0.1	51.1				
L13a	TT**G**^{L}GGAGTAGC	T**G**^{L}G	−72.3 ± 2.5	−194 ± 8	−12.1 ± 0.1	53.8	2.6	0.4 ± 2.8	3.0 ± 8.5	−0.54 ± 0.16
L13b	TTG**G**^{L}GAGTAGC	G**G**^{L}G	−74.0 ± 2.7	−198 ± 8	−12.6 ± 0.2	55.9	4.8	−1.4 ± 3.0	−0.9 ± 9.0	−1.09 ± 0.18
L13c	TTGG**G**^{L}AGTAGC	G**G**^{L}A	−75.2 ± 1.4	−202 ± 4	−12.7 ± 0.1	55.9	4.8	−2.6 ± 1.9	−4.6 ± 5.9	−1.16 ± 0.11
L13d	TTGGG**A**^{L}GTAGC	G**A**^{L}G	−76.9 ± 1.4	−206 ± 4	−13.0 ± 0.1	56.9	5.7	−4.3 ± 2.0	−9.1 ± 5.9	−1.47 ± 0.12
L13e	TTGGGA**G**^{L}TAGC	A**G**^{L}T	−69.4 ± 1.9	−184 ± 6	−12.1 ± 0.1	54.8	3.7	3.3 ± 2.4	12.5 ± 7.2	−0.60 ± 0.14
L13f	TTGGGAG**T**^{L}AGC	G**T**^{L}A	−72.4 ± 2.1	−193 ± 6	−12.6 ± 0.1	56.3	5.1	0.2 ± 2.5	4.2 ± 7.6	−1.07 ± 0.15
L13g	TTGGGAGTA^{L}GC	TA^{L}G	−71.8 ± 1.6	−192 ± 5	−12.3 ± 0.1	54.9	3.8	0.9 ± 2.1	5.3 ± 6.3	−0.76 ± 0.12
L13rv	GCTACTC**C**^{L}CAA	C**C**^{L}C	−70.9 ± 2.2	−189 ± 7	−12.2 ± 0.1	54.8	3.7	1.7 ± 2.6	7.8 ± 7.8	−0.68 ± 0.15

Table 1 (Continued)

LNA-substituted DNA oligonucleotides			results from absorbance melting curves				changes due to LNA substitution			
name	sequence (5′ to 3′)	length, trinuc	$\Delta H°$ (kcal/ mol)	$\Delta S°$ (e.u.)	$\Delta G°_{37}$ (kcal/mol)	T_m (°C)	ΔT_m (°C)	$\Delta\Delta H°$ (kcal/mol)	$\Delta\Delta S°$ (e.u.)	$\Delta\Delta G°_{37}$ (kcal/mol)
D14	CTAAATAGCG	10	-67.3 ± 1.7	-187 ± 6	-9.14 ± 0.03	40.6				
L14a	CTA**L**AATAGCG	T**AL**A	-61.8 ± 2.1	-170 ± 7	-9.17 ± 0.04	41.1	0.5	5.5 ± 2.7	17.8 ± 8.6	-0.03 ± 0.05
L14b	CTAA**L**ATAGCG	A**AL**A	-67.2 ± 2.0	-185 ± 6	-9.73 ± 0.05	43.4	2.8	0.1 ± 2.7	2.2 ± 8.4	-0.59 ± 0.06
L14c	CTAAA**L**TAGCG	A**AL**T	-65.4 ± 2.0	-180 ± 6	-9.56 ± 0.05	42.7	2.1	1.9 ± 2.6	7.3 ± 8.3	-0.42 ± 0.05
L14d	CTAAAT**L**AGCG	AT**L**A	-66.4 ± 1.4	-182 ± 4	-10.03 ± 0.04	44.9	4.3	0.8 ± 2.2	5.5 ± 7.0	-0.89 ± 0.05
D15	TGCACGCTA	9	-49.0 ± 1.4	-125 ± 4	-10.1 ± 0.1	48.2				
L15a	TG**CL**ACGCTA	G**CL**A	-48.1 ± 1.5	-121 ± 5	-10.5 ± 0.1	51.0	2.8	0.9 ± 2.0	4.1 ± 6.2	-0.36 ± 0.08
L15b	TGCA**L**CGCTA	C**AL**C	-53.7 ± 1.8	-139 ± 6	-10.5 ± 0.1	49.8	1.6	-4.7 ± 2.3	-13.8 ± 7.0	-0.42 ± 0.10
L15c	TGCAC**GL**CTA	C**GL**C	-53.5 ± 1.6	-138 ± 5	-10.9 ± 0.1	52.1	3.8	-4.5 ± 2.1	-12.1 ± 6.5	-0.77 ± 0.10
D16	ATTTGACTCAG	11	-76.9 ± 1.7	-214 ± 5	-10.7 ± 0.1	46.5				
L16a	AT**TL**TGACTCAG	T**TL**T	-69.0 ± 2.4	-188 ± 8	-10.8 ± 0.1	48.3	1.7	7.9 ± 3.0	25.9 ± 9.2	-0.12 ± 0.12
L16b	ATTT**GL**ACTCAG	T**GL**A	-66.8 ± 1.8	-181 ± 6	-10.6 ± 0.1	47.6	1.0	10.1 ± 2.4	32.3 ± 7.5	0.09 ± 0.09
L16c	ATTTGA**L**CTCAG	G**AL**C	-67.1 ± 1.7	-181 ± 5	-11.0 ± 0.1	49.5	2.9	9.8 ± 2.4	32.7 ± 7.4	-0.30 ± 0.10
L16d	ATTTGA**CL**TCAG	A**CL**T	-66.0 ± 2.6	-176 ± 8	-11.3 ± 0.1	51.1	4.6	11.0 ± 3.1	37.2 ± 9.5	-0.58 ± 0.14
L16e	ATTTGACT**CL**AG	T**CL**A	-72.3 ± 1.4	-197 ± 4	-11.1 ± 0.1	49.3	2.8	4.6 ± 2.2	16.4 ± 6.8	-0.46 ± 0.09

[a] Each LNA and DNA top strand shown was hybridized to the complementary DNA bottom strand All changes due to LNA were calculated using non-rounded values of the thermodynamic parameters for individual oligonucleotides. Adapted with permission from ref 45. Copyright 2004 American Chemical Society

Table 2. Average Stability Increments for Different LNA Bases[a]

		average thermodynamics parameters for each LNA nucleotide[b]			
		$\Delta\Delta H°$ (kcal/mol)	$\Delta\Delta S°$ (cal/mol K)	$\Delta\Delta G°_{37}$ (kcal/mol)	ΔT_m (°C)
LNA-A	avg ± std dev	3.64 ± 5.04	12.60 ± 15.09	-0.27 ± 0.49	2.11 ± 1.30
	range observed	-4.71 to 12.93	-13.84 to 39.36	-1.47 to 0.75	-0.28 to 5.74
LNA-C	avg ± std dev	1.93 ± 5.52	8.74 ± 16.44	-0.78 ± 0.48	4.44 ± 1.46
	range observed	-8.05 to 11.62	-21.45 to 37.18	-1.72 to 0.35	2.25 to 7.26
LNA-G	avg ± std dev	0.74 ± 3.99	4.17 ± 11.51	-0.56 ± 0.54	2.83 ± 1.75
	range observed	-6.07 to 10.68	-13.70 to 32.28	-1.90 to 0.83	-0.62 to 6.88
LNA-T	avg ± std dev	1.04 ± 4.53	5.33 ± 13.53	-0.62 ± 0.44	3.21 ± 1.41
	range observed	-7.97 to 10.22	-22.01 to 33.11	-1.22 to 0.57	0.62 to 6.17

[a] The effect of LNA substitution on duplex stabilization is represented as the difference in the $\Delta H°$ and $\Delta S°$ for hybridization between LNA-containing and natural DNA, $\Delta\Delta H° = \Delta H°_{LNA/DNA•DNA} - \Delta H°_{DNA•DNA}$ and $\Delta\Delta S° = \Delta S°_{LNA/DNA•DNA} - \Delta S°_{DNA•DNA}$. In terms of $\Delta\Delta G°_{37}$, the average stability increments are ordered $A^L \ll G^L < T^L < C$.[45] [b] Data are averaged over the entire combined data set of 100 oligonucleotides (ref 45).

modified oligonucleotides containing a single LNA substitution in proximity to different neighboring bases. The data has been averaged to give the relative stability increments for each LNA base. Estimation of $\Delta\Delta H°$, $\Delta\Delta S°$, and $\Delta\Delta G°_{37}$ increments attributable to LNA substitution allowed precise prediction of the effect of an LNA substitution on the stability of LNA-modified duplexes based on the nearest 5′ and 3′ neighbors flanking the modification, and also showed that the effect of LNA substitution on duplex stabilization is largely governed by the sequence of neighboring bases. In general, LNA pyrimidines are known to confer more stability than LNA purines, with the order being $A^L \ll G^L < T^L < C^L$ in terms of $\Delta\Delta G°_{37}$ (Table 2). However, there is substantial sequence dependence for each LNA base, and this must be considered in making accurate predictions.[45] Strong enthalpy−entropy compensation was observed across different sequences, showing that the origin of stability is either enthalpy or entropy, but not both at the same time.[45] Another report[46] made use of a 2′-*O*-methyl substituted RNA heptamer to investigate the position-dependent effects of a single LNA substitution on the stability of the heteroduplexes, and accordingly, several principles for the design of 2′-*O*-methyl RNA/LNA chimeras for hybridization to RNA were formulated. The effects of LNA substitutions as a function of sequence context in the middle of the heptamer duplex were investigated and found to be approximately additive when LNA nucleotides are separated by at least one 2′-*O*-methyl nucleotide. Further, the Individual Nearest Neighbor Hydrogen Bonding (INN-HB) model, commonly used for stability predictions for RNA/RNA duplexes, was employed to predict the stability of 2′-*O*-methyl RNA/RNA duplexes. The same model furnished parameters for the stability enhancements of 2′-*O*-methyl RNA/RNA duplexes by substitution of LNA nucleotides internally and/or at the 3′-ends when the LNAs are separated by at least one 2′-*O*-methyl nucleotide.[46] Our own data[47] on hybridization thermodynamics of a series of LNA-substituted octamers showed that the net effect of the LNA modification on enthalpy−entropy compensation is position-dependent, since it is influenced by the neighboring bases. In comparison with an unmodified duplex, the formation of an LNA-modified duplex is associated with a higher counterion uptake and relatively a lower uptake of water molecules. This is attributed to an LNA-mediated structural change in the helical geometry to an A-type conformation that possesses a higher charge density but is less hydrated than the B-type conformation of the unmodified duplexes.[47] Furthermore, hybridization kinetics using stopped-flow study[48] showed that the extra stability of LNA-modified duplexes mainly stems from the slower dissociation rate constants of the two strands of the duplex.

2.3.3. Conformation

The structures of LNA mixmer•RNA and LNA mixmer•DNA duplexes have been characterized using NMR, CD spectroscopy, and X-ray crystallography. In general, the hybrids retain features common for the native nucleic acid duplexes, that is, the usual Watson and Crick base pairing, nucleobases in anti-orientation, base stacking, and a right-handed helical conformation. Structural characterization of three 9-mer LNA/DNA•RNA hybrids where the LNA/DNA mixmers contained one, three, and nine LNA monomers revealed increasing A-like conformation in the hybrid as the LNA content of the modified strand was increased.[49−51] This is attributed to the LNA-induced structural perturbation of the DNA nucleotides in the LNA strand to attain an N-type sugar pucker, which contrasts with the equilibrium between the N- and S-type sugar conformations in the native DNA•RNA duplex (Figure 5). NMR studies describe these

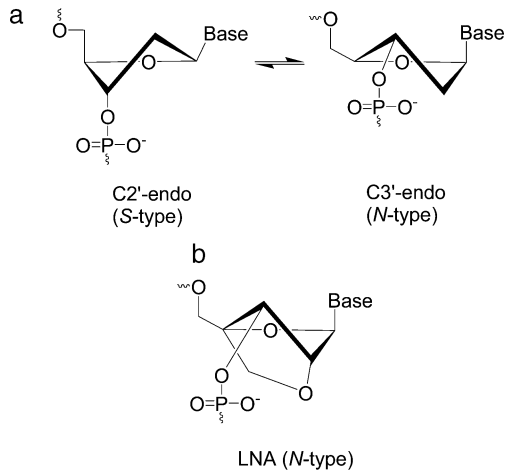


Figure 5. (a) The C2′-endo−C3′-endo sugar ring equilibrium present in nucleic acids. (b) The molecular structure of locked nucleic acid (LNA), which shows the locked C3′-endo sugar conformation.

effects as local, wherein only the flanking nucleotides of the modifications are affected. X-ray crystallography, however, suggested that incorporation of a single modification in a dodecamer DNA stand produced a global effect where all the nucleotides exhibited an N-type sugar pucker.[52] Further, the NMR data showed that the increase in the population of N-type sugar puckers in the hybrid forces an A-like geometry which progressively increases with an increased LNA content.[49−51] However, with the incorporation of three LNA monomers in the nonamer, a saturation level was achieved with respect to structural changes in the sugar conformation. This correlates well with the observation that the increase in helical thermostability per LNA monomer reach a maximum in LNAs containing 50% or less of LNA monomers.[49] This trend was also evident when LNAs were incorporated in a dsDNA duplex, where the inclusion of LNA monomes led to a change in sugar conformation of all the remaining unmodified nucleotides, except for terminal sugars, toward to an N-type conformation.[49] The B conformation of the DNA duplex was thus compromised with the resultant acquisition of A conformation.[52−55]

Similar studies have been performed for 9- and 10-mer α-L-LNA/DNA•DNA duplexes which, in stark contrast to LNA/DNA•DNA duplexes, adopt S-type sugar puckers and retain the B-type helix feature[57,58] (Figure 6). The same α-L-LNA/DNA chimera, when hybridized to RNA, imparts a

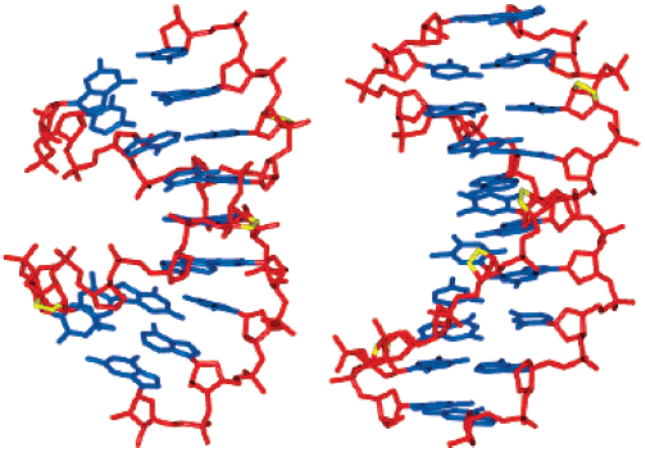


Figure 6. High-resolution NMR structures of LNA•RNA and α-L-LNA•DNA duplexes. The LNA/DNA mixmer•RNA duplex at left adopts an A-type conformation,[49] whereas the α -L-LNA/DNA mixmer•DNA duplex at right adopts a B-type conformation.[58] The mixmer sequence in both the cases is d(5′-CTGATATGC-3′), where each 'T' is modified LNA or α-L-LNA nucleotide, respectively. Reprinted with permission from ref 56. Copyright 2004 American Chemical Society.

helical geometry intermediate between A- and B-type helixes in such a way that the global structure remains very similar to a native unmodified DNA•RNA helix. Therefore, an α-L-LNA mimics a deoxyribonucleotide in both DNA•RNA hybrids and dsDNA duplexes, thus, leaving the global structure unperturbed yet conferring a substantial elevation in the duplex stability.

2.3.4. Triplex-Forming Oligonucleotide (TFO)

The specific recognition by triplex-forming oligonucleotide (TFO) of homopurine−homopyrimidine tracts in duplex DNA provides an attractive strategy for genetic manipulation and is of interest in medicinal chemistry and biotechnology (Figure 7). In a target DNA duplex, the TFO lies in the major groove where it interacts with the purines through Hoogsteen hydrogen bonding. Thus, a TFO can work as an effective antigene agent if capable of interfering with the target gene expression.

Figure 7. Triplex-forming oligonucleotide (TFO) placed in the major groove of the DNA duplex.

However, the extreme instability of pyrimidine motif triplex DNAs at physiological pH limits their application *in vivo* because TFO binding is strongest at a pH low enough to ensure protonation of the cytosines. Systematic studies with different LNA constructs as TFOs have shown that incorporation of one LNA monomer centrally in a TFO leads to a significant increase in its binding affinity ($\Delta T_m > +10$ °C).[59−61] Increasing the number of LNAs in the TFO raises the T_m at pH 6.6 by as much as 4.3−5.0 °C per modification. The presence of LNA modifications in a TFO, thus, leads to increased stability of the triplex at low pH and, also, promotes triplex formation at/near physiological pH.[59−61] This increased stability, which is caused by the increased rigidity of LNA-modified TFO in the free state, causes a significant 20-fold increase in the binding constant at neutral pH.[60] Molecular mechanics modeling studies show the entropy-driven (or less enthalpy-driven) nature of triplex formation by LNA containing TFOs, as compared to unmodified triple helixes.[61] The unusual high puckering amplitude of the LNA residue would exclude effective base stacking if the base pairs were to remain at the center of the helical axis. Consequently, a substantial displacement of the bases of the LNA-containing strand in the groove occurs to relieve steric hindrance between base and sugar moieties and to ensure base stacking.[61] Further, kinetic data ascribes the increase in binding constant at neutral pH to a decreased dissociation rate constant.[60] The binding affinity of TFO can be further increased if it is conjugated to an intercalator such as acridine, which facilitates its interaction with the target DNA strand. In simulating physiological conditions, *in vitro* data indicated a strong DNA binding efficiency for both the acridine-conjugated and -unconjugated 16-mer T,C/LNA; the dissociation constant (K_d) of the former was 200 times smaller than that of the unconjugated counterpart. Further, with high triplex stability, both pyrimidine-rich TFOs exhibited inhibition of the expression of the target gene *in vivo*, but the expression of the mutated gene remained unaffected. The observation suggested that both LNA modification and the intercalator increased TFO activity *in vivo* without affecting its sequence specificity. Furthermore, hybridization studies show that an increase in pH led to a faster dissociation constant of the TFO, while with an acridine-conjugated LNA/TFO, a stable triplex could be formed at neutral pH.[62]

Detailed investigations of sequence- and pH-dependent effects of TFOs containing LNA have allowed formulation of guidelines for the optimum design of LNA-based TFOs.[63] The LNA−DNA residues of the TFO should alternate, and the number of thymine LNA residues in the TFO should be maximized for stable triplex formation.[61] The hybridization properties of TFOs containing G-LNA nucleotides have also been characterized and have been shown to exhibit enhanced stability as compared to the isosequential phosphodiester and to a pyrimidine LNA-TFO directed against the same target. It was shown that TFOs containing G-LNA nucleotides produced an inhibitory effect even under conditions where the parent phosphodiester failed to act.[63]

Finally, the potential of α-L-LNA to form a stable triple helix has also been investigated using TFOs of different designs. α-L-LNA could form a stable TFO at physiological pH, and unlike fully modified LNA TFO, a fully modified α-L-LNA could mediate stable triplex interaction at pH 6.8, albeit with lower stability than partially modified TFOs. Compared to the unmodified triplexes, the enthalpy of formation of modified triplexes was negative, suggesting that different kinds of hydrogen bonding and/or stacking interactions occur in α-L-LNA-modified triplexes than in unmodified triplexes. Finally, some generalizations were formulated for the optimum design of α-L-LNA TFOs: modification at every third or fourth residue of the TFO is favored, as is the optimization of the number of cytosines of α-L-LNA TFOs.[64]

2.3.5. RNase H Activation

Among the various gene-silencing strategies adopted by the modified oligonucleotides, RNase-H mediated cleavage of the target mRNA is one of the most potent and commonly used, since the enzyme is found in all cell types (Figure 8).

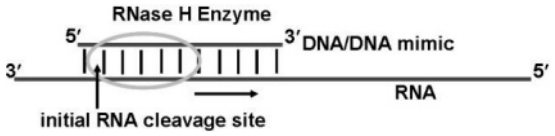


Figure 8. Illustration of the model of nuclease RNase H digestion of RNA•DNA hybrids. The vertical arrow indicates that the enzyme will cleave the RNA strand further toward its 5′-end until the enzyme senses the end of the hybrid.

In fact, the ability of an antisense oligonucleotide (AON) to induce RNase H activation is an important consideration in judging its potential as a therapeutic agent. Among the various AONs that display promising affinities for RNA, only a handful form RNA hybrids that are recognized and cleaved by RNase H. LNA-based oligonucleotides serve as potent antisense agents, since they have excellent binding to their respective complementary strand and are able to block RNA processing enzymes. They also exhibit significant RNase H activation, but 'only' when used in the form of so-called gapmers. An LNA gapmer construct is composed of a central core of DNA or phosphorothioate DNA flanked by LNA nucleotides from both ends. While RNase H binds to both dsDNA and DNA•RNA hybrids, it recognizes the latter to initiate RNA hydrolysis.

Studies on RNase H recruitment by LNA gapmers and LNA mixmers (LNA-monomers interspersing DNA-monomers) have yielded conflicting results: In one case, both the LNA/DNA/LNA gapmer containing a hexanucleotide DNA gap and an LNA/DNA mixmer with six DNA and nine LNA interspersed have been shown to elicit RNase H activation, similar to the corresponding DNA and phosphorothioate analogues.[21] Subsequent studies, however, showed contradictory results with the mixmers. In one study, no RNase-mediated cleavage was observed with an 11-mer LNA/DNA mixmer or with a fully modified 11-mer LNA.[65] Yet another study revealed that a contiguous stretch of six DNA monomers within an LNA/DNA oligonucleotide was necessary to restore 65% of the RNase H activation and that a gap of 7−8 DNA monomers completely restores the activity. In contrast, no cleavage could be observed for LNA/DNA mixmers when bound to target mRNA.[42] The failure of LNA oligonucleotides to elicit RNase H activity when in mixmer design could be attributed to the LNA-induced conformational change in the helix geometry. LNA-based oligonucleotides tend to adopt an A-type helix geometry, while RNase H requires as its reognition element a minor groove width intermediate between that of an A-type RNA and a B-type DNA helix.[66] The change toward A-type character is much more significant when an LNA mixmer hybridizes to RNA compared to when an LNA gapmer hybridizes to RNA. The

excessively increased A-type character of an LNA mixmer•RNA hybrid prevents it from recruiting RNase H, while the minor groove width of an LNA gapmer•RNA hybrid is quite compatible with the enzyme and thus allows efficient RNase H recruitment. Once the RNase H activity is restored, the efficiency of an oligonucleotide in eliciting RNase H cleavage correlates with its affinity for the target RNA (i.e., LNA > 2′-*O*-Me-RNA > DNA > PS-DNA).[42] Another noteworthy finding was the ability of α-L-LNA to mediate RNase H activation, which proved that the locked furanose conformation is not incompatible with RNase H activity. Since, α-L-LNA is a deoxyribonucleotide mimic, the minor groove width (average 8.4), the intra-strand phosphorus distances, and several helical parameters of an α-L-LNA•RNA hybrid resemble that of an DNA•RNA duplex. The α-L-LNA•RNA hybrid could, therefore, elicit RNase H cleavage of the RNA strand, albeit the cleavage rate is significantly lower than an unmodified DNA•RNA hybrid.[57] A comparison of RNase H recruitment activity of β-D-LNA (LNA) and α-L-LNA showed that unlike β-D-LNA, an α-L-LNA can elicit RNase H activity even in a non-gapmer design.[67] Further, RNase H cleavage kinetics studies showed that among LNA gapmers, DNA, phosphorothiate, 2′-*O*-methyl/DNA, and LNA/DNA oligonucleotide, the highest efficacy for inducing RNA cleavage is exhibited by the LNA gapmers (Figure 9). The study also revealed a correlation between T_m and the kinetics of RNase cleavage wherein an increase in T_m by the introduction of modified nucleotides raised the rate of cleavage.[42]

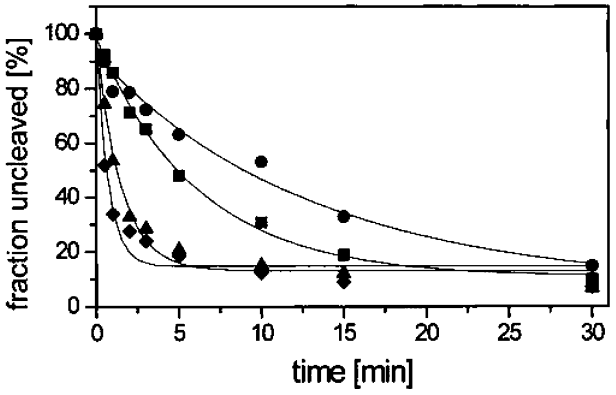


Figure 9. RNase H cleavage kinetics for differently modified oligonucleotides. Target mRNA and equimolar amounts of all DNA (squares), phosphorothiate (circles), chimeric LNA/DNA (diamonds), and 2′-*O*-Methyl/DNA (triangles) oligonucleotides, respectively, were incubated in the presence of RNase H. The fraction remaining uncleaved as determined from aliquots taken from the sample at appropriate time points is plotted as a function of time. The rate of RNase H-mediated cleavage was observed to be the largest for LNA-modified oligonucleotides (diamonds). Reprinted with permission from ref 42. Copyright 2002 Oxford University Press.

2.3.6. Nuclease Resistance and Serum Stability

The sensitivity of unmodified AONs to nucleolytic degradation in biological media limits their use in cell cultures and animals. Thus, the need for an oligonucleotide modified to the extent that nucleases do not recognize it as a substrate becomes a prerequisite for most antisense experiments. In this context, LNAs are preferred over other modified nucleotides because of their high biological stability and low toxicity. Serum stability assays done on LNA/DNA chimeras of different designs revealed that stability is sequence-dependent. Biostability issues for LNA-based oligonucleotides have been addressed for both exonucleases and endonucleases. End-protection with LNA increases the half-life of oligonucleotides in human serum significantly more than the corresponding phosphorothioate DNA gaps and 2′-*O*-methyl flanks.[42] Serum stability assays show that LNA/DNA/LNA gapmers are much more stable than DNA alone, and also that LNA/DNA mixmers are even more resistant to nuclease digestion.[21] The end-capped LNA gapmers are, however, less stable to serum endonucleases. Susceptibility tests with S1 endonuclease indicate that fully modified LNA and fully modified α-L-LNA are very stable against S1 endonuclease digestion, whereas the presence of a DNA gap makes them prone to cleavage.[67]

Significant resistance to exonucleolytic activity is observed on blocking the 3′-end of an oligonucleotide with two LNA monomers (LNA/DNA mixmers). On the other hand, little or no protection is afforded by one penultimate LNA or by a single LNA nucleotide in the middle of the sequence.[68,69] Complete protection against 3′-exonuclease snake venom phosphodiesterase (SVPD) is conferred by a fully modified LNA.[67] Increased stability against SVPD is also observed for an α-L-LNA nucleotide positioned penultimate to the 3′-end, and this stability is further impoved for an oligonucleotide which also contains a number of internally positioned α-L-LNA nucleotides (α-L-LNA /DNA mixmers).[70]

Polymerases usually display some nuclease activity, and their effects on LNA oligonucleotides have been observed during primer extension with a few LNA monomers near or at the 3′-end.[71] It has been observed that positioning of a single 3′-terminal LNA nucleotide significantly lowers the degradation by the 3′ → 5′ proofreading exonuclease activity of the polymerases Pfu and Vent, while the exonuclease function of T7 DNA polymerase and of Klenow Fragment is not significantly impeded. In contrast, a single LNA nucleotide in the penultimate position provides complete resistance to all nucleases tested in this study.[71]

Serum stability assays have been done with both single- and double-stranded oligonucleotides. Under the experimental conditions used in one of the studies involving double-stranded oligonucleotides,[72] presence of one or two terminal LNA bases outside the NF-κB (a transcription factor) binding sequence is sufficient to confer maximal resistance to DNase I (NF-κB(a) and NF-κB(b)) (Figure 10). However, internally positioned LNA substitution caused no significant further improvement in endonuclease-resistance (NF-κB(c), NF-κB(c+b), and NF-κB(b+c) (Figure 10). Resistance to DNase I (a double-strand-specific endonuclease) degradation of NF-κB(a) and NF-κB(b) is of particular interest, since this nuclease is known to recognize certain helical parameters of its DNA substrate, such as groove width and flexibility.[73,74] The fact that the presence of terminal LNAs is able to confer protection against DNase I digestion suggests that LNA−LNA base pairing at the terminal ends probably induces changes in oligonucleotide phosphate-backbone geometry that perturb the interactions between the enzyme and the target DNA in a way that the substrate is no longer prone to cleavage by the enzyme. Interestingly, this perturbation in the oligonucleotide geometry does not affect interaction between the protein NF-κB and the LNA/DNA/LNA copolymers. Further, Bal 31 exonucleolytic degradation showed that at least two terminal LNA bases per strand were required to confer protection from exonuclease digestion. However, significant stabilization in terms of Bal 31 exonucleolytic

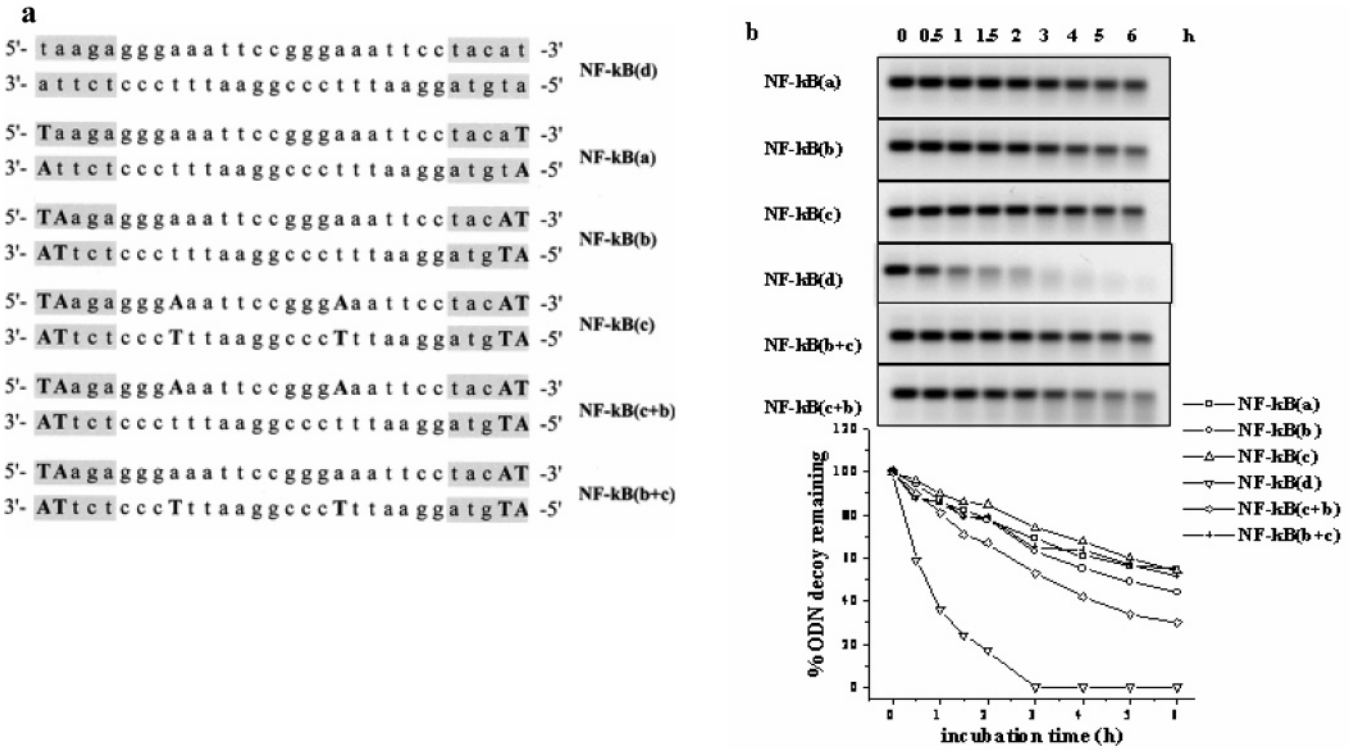


Figure 10. (a) LNA oligonucleotides (20-mer) of different designs consisting of two copies of NF-κB binding sequences, which have been extended at both ends with unrelated extra sequences of 5 nt (in gray). A 30-mer phosphodiester oligonucleotide with same sequence was used as control. LNA substitutions are indicated in bold upper case letters. Reprinted with permission from ref 72. Copyright 2002 Oxford University Press. Figure 10. (b) Susceptibility to DNase I degradation of LNA-modified oligonucleotides, [NF-κB(a), (b), (c), (c+b), and (b+c)] and control phosphodiester [NF-κB(d)], incubated for different lengths of time with 0.5 U/mL DNase I and then submitted to electrophoretic separation on 2.5% (w/v) agarose gels. NF-κB(a) and NF-κB(b) oligonucleotides, containing one or two terminal LNA bases, conferred maximum stabilization against the DNase I endonuclease activity. Reprinted with permission from ref 72. Copyright 2002 Oxford University Press.

cleavage could be achieved with internal LNAs, but only when they were present in both strands.[72]

2.3.7. Delivery into Cell

LNA oligonucleotides have a high potential for use in diagnostics and therapeutics provided that they penetrate the cell membrane barrier and successfully interact with the intracellular target site. Delivery concerns are especially important when oligonucleotides with new chemistry are characterized and compared with others. Unlike PNAs and morpholinos, the charged phosphate backbone of LNA allows its convenient delivery to cells using routinely employed conventional methods for oligonucleotide transfection. Both carrier- and non-carrier-based methods have been investigated as mediators of efficient delivery into the MCF-7 cell line. It was found that non-carrier methods such as scrape loading and electroporation, damage cells and are thus not useful. Five other carrier systems have been successfully employed, namely, FuGENE 6, lipofectin, lipofectAMINE 2000, PolyFect, and Oligofectamine,[21,75] and among these, lipofectAMINE 2000 proved to be the most efficient, allowing delivery of PS, LNA/PS gapmers, or fully modified LNA oligonucleotides to the nuclei of 60−80% of the MCF-7 cells. However, these oligonucleotides varied greatly in their pattern of compartmentalization. Phosphorothioate oligonucleotides were distributed uniformly throughout the nucleus, whereas both LNA/PS and fully modified LNA accumulated in the nucleoli.[40] Similar studies with fully modified LNA oligonucleotide delivered by Lipofectine showed primarily nuclear localization.[20] This pattern was consistent with the high affinity of LNA for RNA. Preferential localization of LNA/DNA chimera is consistently observed in the rRNA component of rough endoplasmic reticulum.[40] Studies employing delivery by lipofectAMINE and Effectin conducted on HeLa cell lines and fixed DU145E prostate cancer cells showed localization of LNAs in both cytoplasm and nucleus, whereas for CV-1 monkey kidney cells, localization was exclusive to the nucleus.[43] We conclude that LNAs can be efficiently delivered into mammalian cell lines using standard protocols, although the precise localization of LNA may vary with details such as the cell type, the oligonucleotide sequence, the carrier chosen, the transfection duration, and so forth.

2.3.8. In Vivo Toxicity

To qualify as a therapeutic tool, the agent must display high efficacy and low toxicity *in vivo*. To date, most human antisense studies use phosphorothioate DNA analogues. Although markedly resistant to the action of deoxyribonucleases, they are known to mediate nonspecific interactions with proteins because of their polyanionic backbone. This phenomenon contributes to the toxic profile of phophorothioates (fever, hypotension, asthenia, complement activation, thrombocytopenia), thus, limiting their application *in vivo*. In contrast, LNAs are well-tolerated in biological systems. The toxicity of LNAs has been investigated systemically in rodents (Figure 11). Unlike the severe tissue damage caused by phophorothioates in the rat brain (caudate putamen), LNA-containing oligonucleotides were as well-tolerated as their DNA counterparts (Figure 11).[21] LNA-mediated toxic effects have been examined for both LNA chimeras (LNA/2′-*O*-methyl chimeric oligonucleotide)[76] and fully modified LNAs.[77]

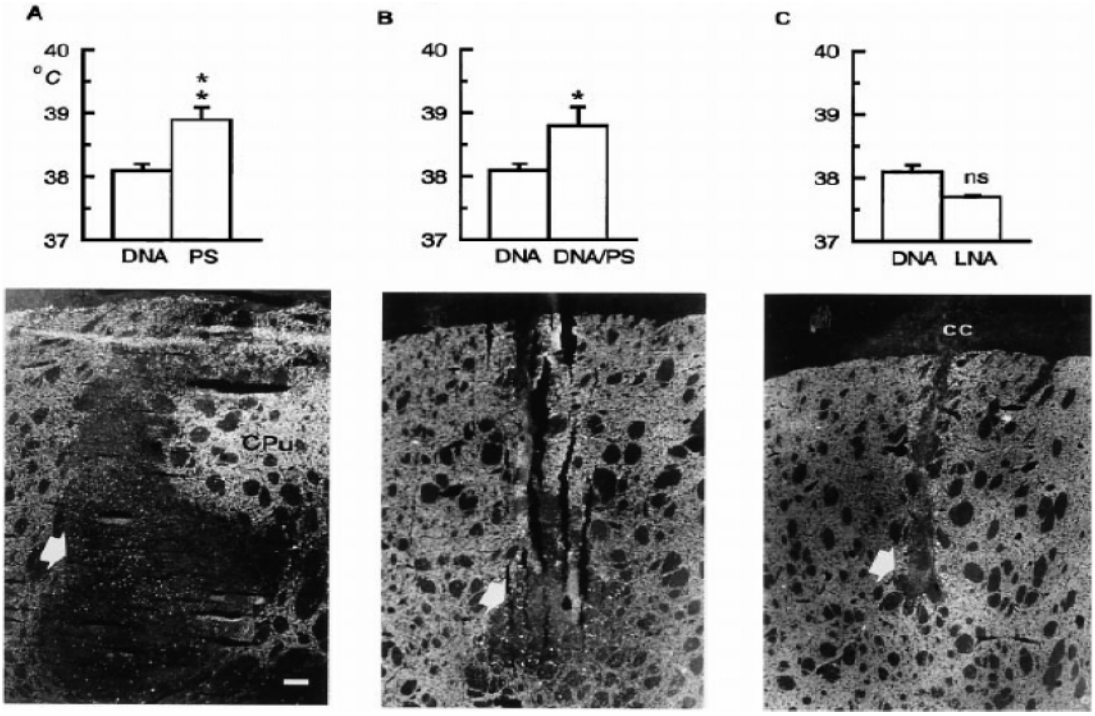


Figure 11. Rat core temperature and the histological examination of rat brain (caudate-putamen) used as markers for toxic manifestations. Panels A−C show the increase in core body temperature (the normal temperature being 37.5 °C) following introduction of (A) phosphorothioates (PS), (B) DNA/phosphorothioate gapmer, and (C) LNA oligodeoxynucleotides in rat brain. Arrows in the fluorescence micrographs show lesions that indicate antisense-induced severe tissue damage. LNA oligonucleotides behaved like their DNA counterparts and exhibited no toxicity, but severe tissue damage was observed for other modified oligonucleotides.[21] Reprinted with permission from ref 20. Copyright 2000 National Academy of Sciences, U.S.A.

The latter have been tested in the murine model system using the serum levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) as markers for toxic manifestations.[77] In both studies, it was found that continuous treatment with LNA oligonucleotide is well-tolerated, and minor toxicity manifests only at doses well above the optimum dose. The low-toxic profile exhibited by LNA-modified oligonucleotides is attributed to the fact that LNA-based AONs, by virtue of their high affinity and specificity, can be reduced to a length shorter than that required by the conventially used DNA AONs. This allows a short length of LNA oligomer to successfully mediate a desired biological effect with only a low degree of binding to serum proteins and, thus, without displaying many of the toxic manifestations that arise merely from the longer polyanionic backbone of the classical AONs.

One of the major concerns for antisense therapy is the recognition of antisense oligonucleotides as foreign invaders in biological systems. Experiments have shown that oligonucleotide fragments containing a two-base sequence, CpG (Cytosine-phosphate-Guanine), present in a correct sequence context, mediate immunostimulatory effects. However, this immune response is triggered only when the CpG sequence is unmethylated. Since unmethylation is common in bacterial DNA and not in mammalian DNA, the immune sysytem considers unmethylated oligonucleotide sequences as bacterial and attacks them.[78] Some phosphorothioate AONs bearing CpG dinucleotides also possess immune modulatory capacities.[79] Since cytosine-LNAs are routinely synthesized as methyl-cytosine-LNAs, it is likely that LNA-CpG motifs are poor immune stimulators. Nevertheless, the effect of LNA substitutions on immune stimulation mediated by an antisense CpG containing LNA/PS/LNA gapmer has been tested.[80] Increasing the number of LNA residues in the flanks or substitutions of CpG nucleobases with LNA reduce or eliminate the immunostimulatory effects of CpG-containing phosphorothioate oligonucleotide. We anticipate this to mean that LNA oligomers *per se* are poor immunostimulators.

3. LNA as a Tool: Implications

3.1. LNA as a Therapeutic

3.1.1. Antisense Agent

3.1.1.1. Targeting mRNA. The concept of antisense agents is not new, and theoretically possesses enormous potential to modulate gene expression at the level of RNA. In practice, however, gene inhibition by employing antisense oligomers has not proven to be robust or reliable, because all available antisense agents used until now suffer from one or more inherent weaknesses.[81] To be highly potent, antisense oligomers need to be highly selective. Among the classes of new reliable antisense molecules, LNA oligonucleotides are obvious candidates for possessing desirable gene silencing properties such as improved hybridization, high specificity, and high nuclease stability for *in vivo* applications. The RNase H activation potential of LNAs in a gapmer design has been successfully used to silence expression of Intracellular adhesion molecule-1 (ICAM-1).[44] ICAM-1, which is expressed on the surface of endothelial cells, is important in cell-to-cell mediated immune response and acts as a receptor for the major group of human rhinoviruses. Targeting of the 3′ untranslated region (3′-UTR) of ICAM-1 mRNA revealed an LNA gapmer containing a nonanucleotide DNA gap to be a potent inhibitor of ICAM-1 expression in HUVEC cells, acting in a dose-dependent and sequence-specific manner.[44] The performance of β-D-LNA in terms of antisense activity, RNase H recruitment, nuclease stability,

and thermal stability has also been evaluated with respect to α-L-LNA. Different gapmers and mixmers designs of β-D-LNA and α-L-LNA were tested. While β-D-LNA exhibited a more potent antisense activity than α-L-LNA, more versatile design possibilities were displayed by α-L-LNA, eliciting RNase H recruitment even in the non-gapmer design.[67]

LNA can also exert its antisense effect by presenting a steric block to the translational apparatus.[40] Different designs of LNA oligonucleotides, for example, fully modified LNA, LNA/DNA chimera, and LNA/DNA/LNA gapmers, were effectively targeted against the 5′-UTR, the region surrounding the start codon, and the coding region of firefly luciferase mRNA, and all of these LNA-based oligonucleotides inhibited luciferase activity. However, they differed in their mechanism of action. Oligomers directed to the translation start site and the coding region required consecutive DNA bases in their backbone to induce RNase-H mediated cleavage, whereas active oligomers targeting the 5′-UTR region blocked ribosome binding to the transcripts and, thus, inhibited translation sterically.[40] The desirable antisense attributes of LNA have been validated *in vivo*, where two different LNA sequences have been used to target mRNA encoding δ type opioid receptors (DOR) in the central nervous system of the rat. Both an LNA/DNA mixmer and an LNA/DNA/LNA gapmer proved to be highly efficacious in reducing the expression of DOR in a dose-dependent manner, with potencies being superior to that of an isosequential phosphodiester, and without any toxic manifestations.[21] The attractive set of properties offered by LNAs in terms of biostability, RNase H activation, lack of toxicity, and potent biological activity prompted exploration into its prospects as an anticancer agent. The efficacy of LNAs in downregulating expression of two endogenous proteins, namely, cyclin-dependent kinase inhibitor p21 and estrogen receptor alpha (ER alpha), both of which are implicated in cancer development, has been compared with standard PS AONs in human breast cancer cell line MCF-7.[75] It was found that an LNA/PS/LNA gapmer caused RNase-H mediated downregulation of the expression of both proteins, while fully modified LNA, LNA/DNA, or LNA/PS mixmers were inactive. A similar study targeting protein kinase C-α (PKC-α) mRNA in lung carcinoma cell line showed greater downregulation of the target by a full-length LNA/PS/LNA gapmer and also by a four-nucleotide truncated LNA/PS/LNA gapmer than the isosequential phosphorothioated AON ISIS 3521.[82] LNA AONs also mediate effective downregulation of anti-apoptotic proteins Bcl-2, Bcl-xL, and survivin in different cancer cell lines.[83,84] Since all known human tumors express either Bcl-2, Bcl-xL, or both, an antisense strategy targeting Bcl-2 and Bcl-xL simultaneously, has broad clinical applicability. The ability of a well-known antisense ODN design of an LNA/DNA/LNA gapmer to inhibit tumor growth by targeting H-Ras mRNA has been successfully demonstrated in both cancer cell cultures and a nude mouse model bearing prostate tumor xenografts.[23] High antisense efficacy was also exhibited by anti-H-Ras ODN containing α-L-LNA. These results indicated that α-L-LNA is a promising member of the LNA molecular family when it comes to antisense applications.[23] LNA-based antisense strategies are also increasingly the subject of *in vivo* studies as well: LNAs have been used successfully to elucidate the molecular mechanisms by which estradiol (E2) acts to reduce sleep and increase activity in rodents,[85] and also to establish the physiological role of the steroid receptor coactivators-1 (SRC-1) in Japanese quail.[86] In another *in vivo* study, a fully modified LNA targeting the RNA polymerase II gene product caused significant reduction of tumor growth in mice at a nontoxic dose of <5 (mg/kg)/day. These findings highlight the superiority of LNA AONs as anti-tumor agents over the isosequential phosphorothioated AONs.[77,87] In another therapeutic application, the antisense LNA has been shown to inhibit translation of Hepatitis virus, which contains within the 5′-UTR region a highly conserved internal ribosome entry site (IRES) that mediates end-independent ribosomal attachment to an internal position in the mRNA. LNA and PNA AONs directed against this site successfully inhibit viral replication by inhibiting IRES-dependent translation.[88]

Finally, the antisense efficacy of LNA has been compared with another gene silencing approach, namely, small interfering RNA. In this study, the potency of an LNA/DNA/LNA gapmer as well as of an isosequential PS and a 2′-*O*-Me gapmer in knocking down the expression of the vanilloid receptor subtype 1 (VR1) was compared with that of siRNA.[89] The potency of the LNA/DNA/LNA gapmer (IC_{50} of 0.4 nM) was found to be manyfolds better than those of the phosphorothioate ($IC_{50} \sim 70$ nM) and the 2′-*O*-methyl/DNA gapmer ($IC_{50} \sim 220$ nM) AONs. Furthermore, siRNA and LNA/DNA/LNA gapmers completely blocked the expression of VR1−GFP at low-nanomolar concentrations. A significant antisense effect was observed for phosphorothioate at concentrations higher than 25 nM, whereas no reduction of gene expression was found for the *O*-Me-modified gapmer (Figure 12). Thus, siRNAs and chimeric LNA/DNA oligonucleotides act as potent antisense agents.[89]

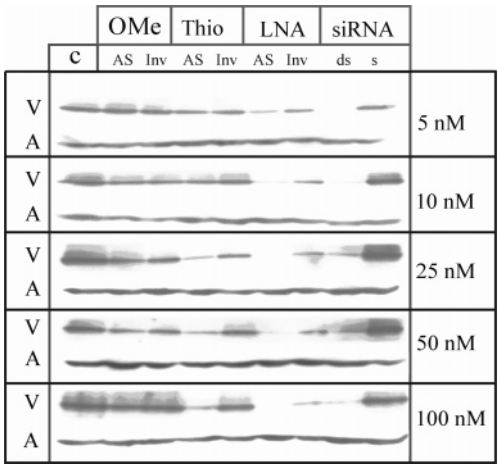


Figure 12. Western blot analysis from cotransfection experiments of the plasmid encoding the VR1−GFP fusion protein and modified antisense (AS) oligonucleotides. OMe-DNA and LNA gapmers, PS, and the siRNA were used at concentrations between 5 and 100 nM. Inverted control oligonucleotides (Inv) (with sequence invert of the AON sequence) for each modification and the sense strand (s) of the siRNA were used as controls. Cells were harvested after 24 h, and Western blots were performed with an anti-GFP antibody. To confirm equal loading of the samples, membranes were reprobed with a monoclonal mouse antibody against actin. V, VR1−GFP band; A, actin band; C, control VR1−GFP expression in the absence of AON. Reprinted with permission from ref 89. Copyright 2003 Oxford University Press.

Lately, the development of the so-called siLNA technology, which involves using LNA in siRNA technology, has been proposed to improve the thermal stability, serum stability, cellular activity, or pharmacokinetic properties of

siRNAs.[90] siRNAs can accommodate quite a number of modifications at both base-paired and non-base-paired positions without significant loss of activity. LNA is quite compatible with the siRNA machinery and substantially increases the biostability and thermal stability of siRNAs without compromising their efficiency.[91] However, for efficient gene inhibition, the content and positioning of LNA substitutions within an siRNA needs to be optimized. It has been observed that the positioning of LNA substitution at the 5′-end of the 'antisense strand' (i.e., the strand with the sequence complementary to the target mRNA) of the siRNA duplex leads to complete loss of acivity, but the 5′-end of the 'sense strand' (i.e., the strand with the sequence identical to the target mRNA) can be modified without loss of activity. Interestingly, activity lost by 5′-end modification of the antisense strand can be recovered by substituting the 3′-sense end, and this effect is even greater if modifications are included in the 5′-sense end.[92] This is attributed to the strand biasing or the functional asymmetry exhibited by the two strands of siRNA duplex, with only one strand being eligible to trigger mRNA cleavage and the other destined to be destroyed. It is proposed that the strand displaying the weakest binding energy at the closing 5′ base-pair is preferentially incorporated in RISC assembly (Figure 1), which subsequently mediates target mRNA cleavage.[93,94] Substitution of LNA at the 5′ antisense end increases the binding energy of its 5′ base-pair and this prevents it from being assembled in RISC. Further compensatory modifications at the 5′ and 3′ sense end can successfully restore the effect. Apart from terminal modifications, certain internal substitutions in the antisense strand might also hamper siRNA activity. These internal positions are the ones that lie close to the site where mRNA cleavage occurs. It is anticipated that the presence of LNA modification at these sites exerts a direct conformational or functional effect on the catalytic site, which subseqently affects siRNA activity.[92] A major concern for the use of siRNA as a genomic tool is the finding that cells might incorporate both strands into the RISC complex. Incorporation of the unwanted, nontarget complementary sense strand leads to the so-called 'off-target' effect that decreases the potency of siRNA by simply lowering the number of RISC complexes loaded onto the antisense strand. LNA substitution in this case is particularly useful as it may help to minimize these effects by acting through two different mechanisms. LNA substitution at the 5′ sense position will encourage strand loading of the antisense strand. Additional internal substitutions in the sense strand might impair its ability to participate in target cleavage once it is loaded in the RNA-induced silencing complex (RISC) assembly.[92] Together, these findings emphasize the potential of LNA to provide the therapeutic benefits of the siRNA technology.

3.1.1.2. LNAzymes. LNA can possibly be used as a potent antisense agent in the DNAzyme-mediated gene-silencing approach. DNAzymes are catalytically active DNA molecules that bind to specific sequences in RNA and act as specific RNA endonucleases by cleaving the RNA phosphodiester backbone. The most commonly used DNAzymes are derivatives of a 31-nucleotide '10-23' oligomer that consists of a 15 nucleotide catalytic core flanked by two binding arms (Figure 13). The binding arms control the specificity of DNAzyme by hybridization to the RNA substrate, whereas the catalytic core cleaves the phosphodiester bond held between the binding arms.[95] Incorporation

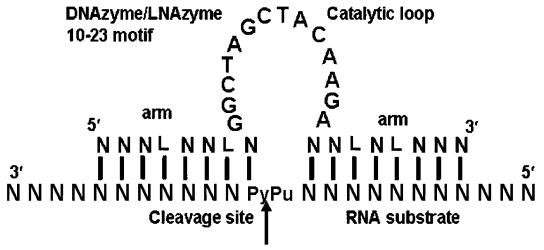


Figure 13. Diagram illustrating the target recognition for 10−23 motif DNA- and LNAzymes. Lines represent Watson−Crick base pairs. The letter 'N' stands for unmodified nucleotides, while 'L' represents LNA-subtsituted monomers.[96] Adapted from ref 96. Copyright 2002 American Chemical Society,

of LNA into the two arms of DNAzyme produces an LNAzyme which confers marked resistance to nucleolytic attack, and simultaneously, increases the cleavage activity, even for highly structured RNAs such as 23S ribosomal RNA.[96] The DNAzyme cleaved only moderately well when present in a 50-fold molar excess of the RNA substrate, whereas stoichiometric amounts of the LNAzyme were sufficient to cleave >50% of the substrate. The bandshift analysis of hybridization complexes run on polyacrylamide gel electrophoresis (PAGE) suggested that the improvement in the cleavage activity was probably due to a higher hybridization affinity of the LNAzyme for the RNA substrate. Similar results were also seen by substituting LNA with α-L-LNA.[96] Optimally designed, LNA-substituted DNAzyme targets the 5′-UTR of human rhinovirus more efficiently than DNAzymes stabilized by other modified nucleotides like phosphorothioate linkages, 2′-*O*-methyl RNA (added to enhance the rate of cleavage), and 3′-3′-inverted thymidine (used to enhance nuclease stability).[97] It was observed that introduction of LNA in the nonanucleotide substrate arm significantly increased the rate of cleavage, albeit only under single turnover conditions. In multiple turnover conditions or in excess of substrate, LNAzyme decelerated the reaction, indicating that it binds to the RNA target too tightly. Cleavage kinetics studies of LNAzymes showed that LNA, irrespective of RNA sizes or structures, boosts substrate hybridization, while no change appears in the substrate scission step.[97] These studies also enabled the design of DNAzymes modified at both binding arms and the catalytic core. It was found that for a heptanucleotide arm with three terminal LNA nucleotides, the initial velocity was increased by a factor of 6, while adding a fourth terminal LNA nucleotide reduces the activity. This can be ascribed to either a modification in the inner part of the binding arm that interferes with the correct folding of the catalytic center or to the requirement of an optimal affinity for high catalytic activity. A correlation of the melting temperatures of the corresponding enzyme/target heteroduplex with the reaction rates demonstrated the pivotal role of the balance between increased substrate affinity and uncompromised product release. Therefore, optimization of the RNA cleavage by LNAzymes requires careful adjustment of the arm length, sequence composition, and number of LNA monomers.[97] In another study, introduction of LNA monomers into the substrate recognition arms of the DNAzyme led to complete cleavage of the previously inaccessible coxsackievirus A21 (CAV-21) RNA at a high catalytic rate, indicating that nucleotides with high target affinity were able to compete successfully with internal structures. This strategy was then adopted to successfully convert two previously inactive DNAzymes into ones highly effective against their respective

target structures.[98] Recently, one effect of LNAzymes was shown to be the inhibition of human vascular smooth muscle cell growth by targeting early growth response-1 (EGR-1) transcript. Even at lower concentrations, LNAzymes are more effective than DNAzymes with respect to *in vitro* substrate cleavage, protein expression, smooth muscle cell proliferation, and regrowth after mechanical injury. Thus, introduction of LNAzymes is indeed a significant step toward the realization of oligonucleotide-based therapeutics with intrinsic endonucleolytic activity.[99]

3.1.1.3. Targeting Noncoding RNA. The propensity of some RNAs (e.g., rRNAs, 5′-UTRs of mRNA, RNAs P, and group I and group II introns) to adopt multiple folded states of similar free energy can be exploited to silence gene expression. The inactive folded states of these RNAs can be trapped kinetically by interaction with short antisense oligonucleotide leading to silencing of the respective RNA function. This method known as "oligonucleotide directed misfolding of RNA" or ODMiR has been demonstrated in *Candida albicans*, a human pathogen, where an 8-mer fully modified AON and a 12-mer LNA/DNA mixmer successfully inhibited group I intron splicing by introducing misfolding.[100] When the intron is transcribed in the presence of these AONs, self-splicing is inhibited by 50% at a concentration of about 150 nM for fully modified AON and 30 nM for LNA/DNA mixmer. On the other hand, the isosequential 12-mer DNA and 2′-*O* methoxy AON displayed higher IC_{50} values. Optical melting experiments of several oligonucleotides with their RNA complements further showed that a stronger base pairing, attributed to LNA-substitution, usually provides a lower IC_{50}. Thus, the high affinity of LNA toward the target strand allowed its access to the highly structured region of RNA.[100]

In another study, LNA-based AON was used to target a cancer-specific ribonucleoprotein telomerase effectively.[43] Telomerase is known to be critical for cancer cell proliferation, since its 11-mer RNA sequence binds to telomeric DNA and thus guides the addition of telomeric repeats from one generation to the next. Targeting this RNA template sequence by an antisense LNA caused a marked reduction in telomerase activity. When they are compared with analogous PNAs, the fully or partially modified LNAs exhibited high affinity recognition for the target template sequence. Furthermore, the fact that different designs of fully modified LNA oligonucleotides or LNA/DNA chimeras effectively inhibited telomerase, while analogous mismatched LNA did not, thereby suggesting that inhibition by LNA/DNA chimera and LNA involves greater stringency.[43] This further suggested that manipulation of LNA length and the backbone substitution pattern can be used to tailor successful telomerase inhibition.

LNA AONs have been employed to sterically block and inhibit the interaction of HIV-1 Tat (Trans-activator of Transcription) protein with an RNA recognition sequence TAR (trans-acting responsive element). HIV-1 transactivation responsive element (TAR) is a 59-nucleotide stem-loop RNA that interacts with HIV transactivator protein (Tat) and other cellular factors to stimulate transcriptional elongation from viral long terminal repeat (LTR). The high degree of sequence conservation in the HIV RNA region makes the TAR stem-loop an ideal target for antisense therapy. Different 12-residue oligonucleotide analogues (fully modified 2′-*O*-methyl, 2′-*O*-methyl chimeras including LNA/2′-*O*-methyl chimeras, and PNAs) complementary to the TAR

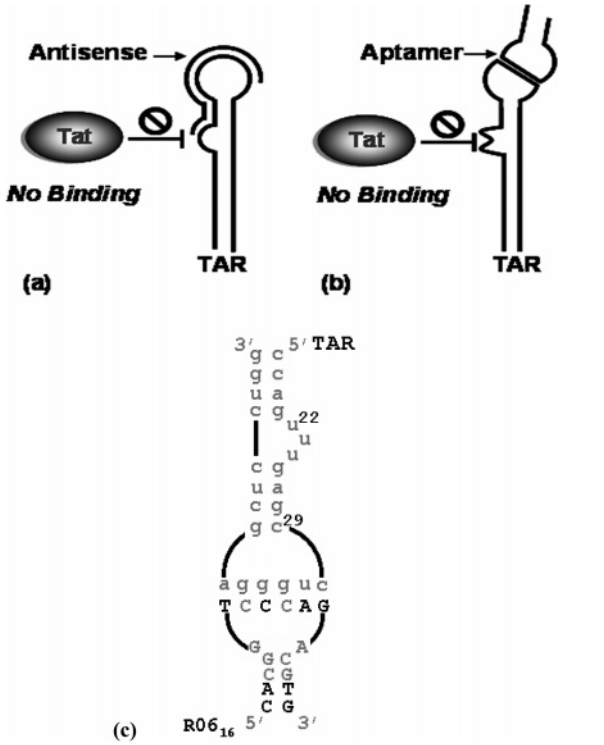


Figure 14. Inhibition of HIV Tat-TAR interaction using (a) antisense and (b) aptamer approach. Presence of an antisense oligonucleotide sterically blocks the binding of Tat to TAR, while the aptamer competes with Tat for binding to TAR and, subsequently, distrupts the native RNA secondary such that TAR is no longer recognized by Tat. (c) Chimeric LNA/DNA aptamers derived from this R06 hairpin RNA interacting with TAR-stem loop of HIV.[104]

apical stem-loop have been shown to inhibit Tat-dependent *in vitro* transcription in HeLa cell nuclear extract equally efficiently (50% inhibition at 100−200 nM) and sequence specifically (Figure 14a).[76,101] In contrast, the antisense effect was poor for short phosphodiesters and phosphorothioates.[102] Cellular studies with HeLa cell using 3′-carboxyfluorscein (FAM)-labeled oligonucleotides allowed intracellular tracking of 12-mer oligonucleotides. In addition to a fully modified 2′-*O*-methyl (OMe) and a 12-mer chimeric AON containing seven 2′-OMe and five LNA residues, additional OMe-FAM derivatives containing a number of phosphorothioate linkages (OMe-FAM-thio) were tested for intracellular inhibition of Tat-dependent transcription. While neither of the OMe-FAM and OMe-FAM-thio derivatives exhibited dose-dependent inhibition, use of a 12-mer FAM-labeled LNA/OMe chimera showed inhibition at submicromolar concentration (70−80% supression at 1 μM) with both sequence and dose dependence.[76] Further, study of the structure−reactivity relationship showed that OMe-oligonucleotides with optimally 40−50% LNA units and a minimum of 12 residues in length were active in the cellular assay, whereas activity in the *in vitro* transcription assay was observed down to 9 residues. The other structural analogues of LNA, namely, the α-L-LNA or 2′-thio-LNA, were also active in repression of *in vitro* transcription, with the former demonstrating that promotion of cellular uptake by LNA is not due to specific sugar conformational effects.[103]

As an alternative to antisense oligomers, the HIV-1 TAR−Tat interaction has also been interrupted by the use of the aptamer approach, in which a short (24-mer) chemically synthesized hairpin RNA (R06) was shown to interact with

TAR through loop−loop interactions (the so-called kissing complex) (Figure 14b). It was found that the chimeric LNA/DNA aptamers (16-mer, designated as R016) derived from this R06 hairpin RNA retained the binding property of the originally selected R06 and successfully competed with binding of Tat peptide to TAR.[104,105] Of the various LNA/DNA aptamers synthesized, a chimeric hairpin with a four base-pair stem containing two LNA pairs at the bottom and four interspersed LNA residues in the eight nucleotide loop (Figure 14c) showed efficient inhibition of TAR−Tat interaction, whereas both the fully modified LNA version of R06 and the chimeric LNA/DNA derivative with an LNA loop and DNA stem or vice versa failed to do so.[104] The selected LNA/DNA aptamer could discriminate between two TAR variants differing only by a single mutation in the loop, whereas an LNA/DNA antisense octamer with the same binding constant as the hairpin chimera could not. Furthermore, unlike the antisense oligomer, the modified aptamer could effectively compete with Tat peptide binding to TAR, even though the binding sites of the two ligands did not overlap each other. This suggests that the aptamer, and not the antisense octamer, upon binding, switches the TAR bulge region, that is, the binding site of Tat, to a structure that is no longer recognized by the Tat peptide.[104]

In another study, the LNA/DNA mixmers and gapmers were used to target another important site of the HIV-1 genome, the dimerization initiation site (DIS). DIS is a stem-loop structure which assists dimerization of the two sense strands of the RNA genome, packaging, and also proviral synthesis. Targeting this site by 9-mer antisense RNA inhibited viral replication, whereas the corresponding antisense DNA did not. However, inclusion of LNA bases in the same antisense DNA augumented the interference of HIV 1−genome dimerization and exhibited the same potent inhibitory effect as did RNA. Concomitant RNase H activation was observed when the terminals of this antisense DNA were substituted with LNA flanks. Interestingly, the 'sense' LNA/DNA mixmer also showed significant inhibitory effect. Since the dimerization interaction involves the coming together of two RNA sense strands, the sense oligonucleotides (LNA/DNA mixmer) can pair with DIS and, thus, likely behave as a competitive inhibitor of the dimerization process.[106]

3.1.2. Current Status: Clinical Trials

Since LNAs possess high hybridization affinity and target selectivity, it is unsurprising that they are strong candidates for antigens and antisense strategies. Their increasing success in cell-line-based experiments has paved their way to clinical trials. The status of LNA in clinical trials have not been reviewed so far; however, pharmaceutical companies are engaged in developing new innovative anti-cancer drugs based on LNA-antisense technology, so as to downregulate genes that are aberrantly activated in many, if not all, types of cancer. One of the most advanced LNA-based drug candidates to have entered a Phase I/II clinical study is being developed for use against Chronic Lymphocytic Leukaemia (CLL). This LNA−AON acts by inhibiting the synthesis of Bcl-2, a key sensor protein that protects cells against apoptosis (programmed cell death). Downregulation of bcl-2 expression resensitizes the cancerous cell to natural apoptotic stimuli and chemotherapeutic drugs that induce apoptosis. LNA-based antagonist technology is also being developed to target and inhibit Survivin, a vital regulator of both apoptosis and cell division, and to inhibit HIF-1a, a key sensor of cellular hypoxia, which responds to hypoxia by transcriptionally upregulating a host of genes that play important roles (such as angiogenesis, apoptosis, cell migration, and metastasis) in the promotion of cancers. Clinically, Survivin and HIF-1a expression are associated with poor prognosis, increased cancer recurrence, and resistance to radiotherapy and cytotoxic drugs. These compounds, thus, have the potential to improve response to therapy in a wide variety of malignant solid tumors and thus become new anti-cancer drugs with multiple modes of action and a broad spectrum of application (www.Santaris.com). The progress of LNA in clinical trials promises to improve the clinical outcome for cancer patients by serving as a potent and safe alternative to the highly toxic chemotherapeutic drugs that are currently used for cancer treatment.

3.1.3. Antigene Strategies

The term "antigene effect" refers to strategies operating at the level of chromosomal DNA to inhibit gene expression at the level of transcription, replication, and other protein−DNA interactions. The "decoy" approach based on the same principle involves the use of dsDNA bearing the consensus binding sequence for a specific transcription factor. Such sequences, when transfected into the cell, interact with the target factor and prevent its binding to the respective promoter, thus, causing a marked reduction of transcriptional activation. This method can be used to antagonize the function of transcription-activating proteins involved in the pathophysiology of human diseases caused by aberrant gene activation or expression.

A panel of double-stranded, modified oligonucleotides (Figure 10a) containing LNA bases at various positions was designed by Crinelli et al.[72] to act as decoys for transcription factor κB (NF-κB). They exhibited high hybridization efficiency and sensitivity, not manifested by PNA oligomers either fully or partially modified with phophorothioate oligonucleotides. The decoy LNA oligonucleotides were recognized by NF-κB, but the efficiency of different competitors for NF-κB binding varied. Inclusion of one or two terminal LNA monomers, in both of the strands and outside of the κB sequence, appreciably increased the protection against nucleases without interfering with transcription factor binding, but positioning of additional LNA substitutions internal to the NF-κB sequence had varying effects on NF-κB binding. This is attributed to the LNA-induced conformational change produced in the helical geometry of the target sequence. The degree of conformational change varied with the extent and position of LNA substitutions. This conformation change further perturbed the extent of binding of NF-κB to the target sequence. Thus, for a few cases where internal substitutions were introduced in both strands, a significant loss of NF-κB binding to the target was seen, but internal substitutions in one strand had minimal effect on NF-κB binding.[72] Positioning of substitutions, whether in one strand or in both strands of the aptamer, can also be optimized to reconcile the biostability with target transcription factor affinity, since there exists a strong correlation between internal LNA substitution position and NF-κB binding. A noteworthy finding was that the diastereoisomeric forms of LNA (α-L-LNA) in place of LNA reverse the adverse effects of LNA on NF-κB binding. With these considerations, the delivery of α-L-LNA-based decoys against NF-κB to the monocytes or macrophages constitute progress

toward the goal of achieving long-term modulation of NF-κB activation and controlled release of proinflammatory mediators in pathophysiological conditions.[107]

Interaction of NF-κB with its target dsDNA is also inhibited by incorporation of LNA in triplex forming oligonucleotides. Presence of LNA increased the stability and affinity of TFO toward dsDNA, allowed triplex formation at pH 7, and prevented binding of transcription factor NF-κB.[26] On similar grounds, it was found that the binding of LNA TFO to the promoter region of multidrug resistant gene 1 (*mdr* 1) reduced the overexpression of drug efflux-related P-glycoprotein in cancer cells.[108]

In addition to TFO and the aptamer antigene approaches, another strategy to attack dsDNA employs a short high-affinity oligonucleotide that binds to the transcription complex. At the beginning of transcription, RNA polymerase unwinds the dsDNA promoter region and exposes a small stretch of ssDNA. During this stage, a short high-affinity 2′-*O*-Me oligoribonucleotide (pentamer) possessing a non-extendable terminal 3′-deoxyribonucleotide can hybridize to the sequence upstream of the promoter region of the template and inhibit transcription by an *in situ* block to elongation.[109] The use of LNA in the 2′-*O*-Me-ribonucleotide pentamer greatly improves this interaction and drastically reduces the efficiency of *Escherichia coli* RNA polymerase at the *lac* UV-5 promoter by nearly 95%.[110]

3.2. LNA in Diagnostics

3.2.1. Probes for Single Nucleotide Polymorphism (SNP) Detection

The exceptionally high binding affinity and sequence specificity of LNA finds significant use in diagnostics where LNA-substituted oligonucleotides are used as probes in several hybridization-based assays such as expression profiling, DNA sequencing, SNP genotyping, and so forth. SNPs are the DNA sequence variations occurring when a single nucleotide in the genome differs between members of a species. SNPs may fall within coding sequences of genes, noncoding regions that may have regulatory effects, or the intergenic regions between the genes. SNPs in the coding region may alter the gene product (polypeptide) and cause several ailments. The traditional time-consuming manual methods of SNP-screening can be automated using LNA technology which relies on a single nucleotide mismatch being discriminated better by LNA oligomers. This high discriminatory power of LNA originating from strong hybridization efficiency and high specificity makes it an ideal tool for use in hybridization-based genotyping assays. Assays based on the ability of allele-specific LNA capture probes to discriminate with enhanced signal-to-noise ratio between matched and mismatched targets have been described. These enzyme-independent genotyping methods have used colorimetry[111−113] and direct fluorescence or fluorescence polarization[114] to signal the hybridization event, and can be run in parallel or multiplexed formats.

LNA primers and templates are read by different polymerases (Klenow and Taq polymerase) and by reverse transcriptase. However, when using LNA-based primers in genotyping, relative positioning of LNAs at and near the 3′-terminus of a primer needs to be considered, since it significantly affects the polymerase-mediated extension.[71] While a terminal LNA nucleotide does not substantially slow primer extension with natural deoxy-NTP substrate, an additional locking at the penultimate position causes significant conformational change in the phosphodiester backbone and consequent repositioning of the terminal nucleotide, which contributes to active site deformation of the polymerases and, ultimately, to the decrease of the polymerization rate by almost an order of magnitude in the case of Klenow fragment. The inclusion of several internal LNA nucleotides leads to conformational changes that induce a transition from B-form to A-form duplex DNA, which may control the upper limit to the number of modified nucleotides that can be included within a primer to be used in enzymatic reactions sensitive to such structural changes.[71]

One of the strategies employed in SNP genotyping is allele-specific PCR (AS-PCR), which is based on positioning of the 3′ base of PCR primer to match one SNP allele and accurately extending the correctly matched primer under stringent conditions. However, natural DNA primers often lead to incorrect priming and, hence, to inaccurate genotyping. Substitution by LNA at the 3′-position of allele-specific PCR primers significantly increases their specificity and reliability. The effects of 3′ LNA incorporation have been investigated for plasmid and human genomic DNA templates, where they exhibited superior allelic discriminatory ability under a wider window of PCR conditions than the corresponding DNA primers.[115] Positioning of LNA at the 3′-position of the primer has also been used to quantify the methylation level of a specific cytosine within a complex genome. The method makes use of methylation-specific real-time PCR with SYBR Green I using one of the primers whose 3′-end discriminates between the methylation states of this cytosine. The presence of a locked nucleic acid at the 3′-end of the discriminative primer provides the specificity necessary for accurate and sensitive quantification, even when one of the methylation states is present at a level as low as 1% of the overall population.[116]

The extraordinary advantage of LNA probes has also been exploited in ELISA-like assays where immobilized allele-specific LNA capture probes detect SNPs in PCR-amplicons from human genomic DNA, for example, factor V Laiden mutation, an apolipoprotein B (apoB) R3500Q mutation, a two-mutation apolipoprotein E, and so forth.[111−113] The screening assays rely on hybridization of biotinylated-PCR-amplicons of the target sequence to the complementary LNA-modified capture probes which have been covalently immobilized on microtiter plates. Subsequently, hybridization is scored colorimetrically using a horseradish peroxidase−anti-biotin Fab conjugate and tetramethylbenzidine, a chromogenic substrate for horseradish peroxidase. The presence of LNA probes allowed efficient and specific interrogation of SNPs in which the hybridization signal from the perfectly matched targets was more than 10 times higher than that from the single-nucleotide-mismatched targets. Further, the conformational fixation of the sugar moiety in the LNA nucleotide enables the design of short LNA capture probes capable of identifying DNA targets differing only by a single nucleotide.[111−113] SNP genotyping within a β-adrenergic receptor is also based on a similar assay in which two differently labeled, allele-specific, LNA-spiked probes compete for hybridization to immobilized, denatured PCR-amplicons.[117]

In addition to solid-phase SNP genotyping, LNA probes have been used in homogeneous (liquid) hybridization for SNP genotyping. Here, fluorescence polarization (FP) values of a fluorescent dye, such as rhodamine/hexachlorofluores,

attached to the LNA probe increases significantly upon hybridization of the probe to the target DNA molecule. In contrast, the presence of single mismatch results in only a small or even no change of this parameter. Based on its high sensitivity and relative simplicity, this assay is suitable for high-throughput screening of SNPs. Furthermore, multiplexing in the assay could be achieved using differentially labeled wild-type- and mutant-specific probes in the same solution to allow simultaneous detection of several SNPs.[114] Homogeneous SNP genotyping assay has also been adopted to screen co-segregating variants of toll-like receptor-4 (TLR4), a member of a large family of transmembrane proteins which are predominantly expressed on monocytes and macrophages and are involved in various diseases and in innate and adaptive immunity. Its variants are associated with a blunted response to inhaled lipopolysaccharides that alters the immune response to pathogens. Detection of variants in this case was made possible by the use of LNA-modified SimpleProbe oligomers on the LightTyper instrument. These probes used a terminal self-quenching fluorophore that changed its properties when stacked with the neighboring base during binding, thereby increasing fluorescence signal, a method that has allowed rapid detection of the wild-type, mutant, or heterozygous samples.[118] LNA-modified, fluorophore-labeled hybridization probes in LightCycler assays have also allowed genotyping of triallelic variant (G2677T/A) in MDR-1 gene. The genotyping results produced by this assay were validated using PCR-RFLP with 100% concordance.[119] Fluorogenic LNA probes have detected factor V Leiden mutation and sickle cell anemia mutation in the β-globin gene using 5′-nuclease assay (Figure 15). The assay pointed out the enhanced discriminatory power of LNA oligonucleotides by outperforming the comparable DNA probes.[120] The use of LNAs in 5′-nuclease assay has also been compared to the routinely used minor groove binder (MGB) probes in terms of their ability to target staphylococcal enterotoxin gene. The study showed equivalent sensitivity and specificity for both of the probes, suggesting that the use of LNA probes provides an attractive alternative to MGB probes for rapid detection of genes of bacterial pathogens.[121]

Recently, LNA probes have been used in a one-step assay for asymmetric PCR and homogeneous hybridization for genotyping HLA-DQBI alleles associated with susceptibility to Type I diabetes. The genotyping of HLA-DQB1 is rather complex, since there is no one single SNP that is distinct from other allele. Despite this, the LNA probes used were very specific and produced no cross-reactivity. It is remarkable that where DNA probes failed to give readable results due to the presence of secondary structure in the PCR product, LNA probes allowed good separation of genotypes.[122]

Polymorphism studies using LNA probes have also allowed association of polymorphism in PGCα-1 (Gly483Ser), a coactivator of numerous transcription factors, with hypertension in Danish White population.[123] Another assay for screening mutant populations which uses the high sensitivity of LNA probes involves Clamped-Probes that allow sensitive detection of minority mutations in a tissue sample containing excess wild-type DNA. Screening, in this case, is greatly facilitated by the use of non-extendable, LNA-containing clamping oligomers (Figure 16). The method is successful in screening for the *Pfcrt* K76T mutation associated with *in vitro* chloroquine resistance[124] and could even detect minority

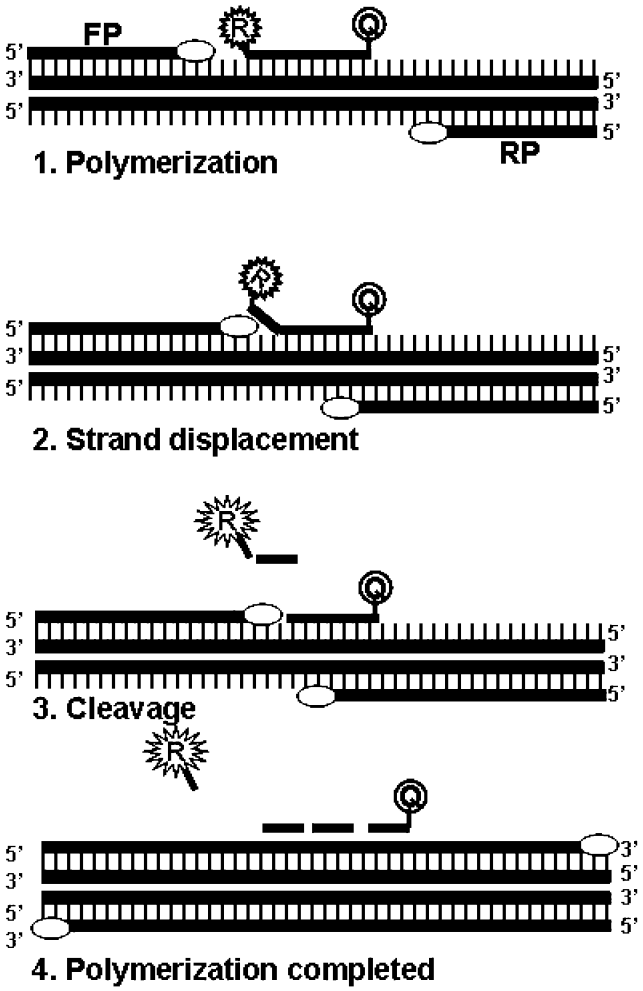


Figure 15. Schematic representation of Fluorogenic 5′- nuclease assay. The method uses dually labeled LNA probe hybridizing within the DNA target sequence bound by PCR primers. In a hybridized state, the reporter fluorescence is suppressed, since it lies in close proximity to the quencher. However, during primer extension, the 5′-nuclease activity of the Taq polymerase cleaves the probe, which results in increased fluorescence of the reporter probe. Mismatches between the probe and target reduce the efficiency of probe hybridization. Furthermore, the enzyme is more likely to displace a mismatched probe reather that cleave it to release the reporter dye. FP, forward primer; RP, reverse primer; R, reporter; Q, quencher; P, fluorogenic LNA probe.

point- and tandem-*B-raf* (an oncogene) mutations in mixtures of mutant and wild-type melanoma.[125] With the use of similar PNA−LNA clamps, screening for genetic heterogeneity in epidermal growth factor receptor (EGFR) has been successfully done to allow the detection of EGFR mutations against a large background of wild-type EGFR.[126]

LNA oligonucleotides have furthermore been successfully applied to FISH (fluorescence *in situ* hybridization) where LNA/DNA chimeras have been used to probe different tandem repeats sequence elements, such as the satellite-2 repeat in heterochromatic regions, the α-satellite repeats in centromeres, and the telomeric repeats. In comparison with DNA probes, LNA probes produced a strong signal, with a short time of hybridization, and detected regions as small as 1−3 kb.[127,128]

Highly efficient LNA probes have opened new avenues for assays based on nucleic acid hybridization. However, quantitative studies on genomes strongly demand biomolecular recognition probes with high specificity and

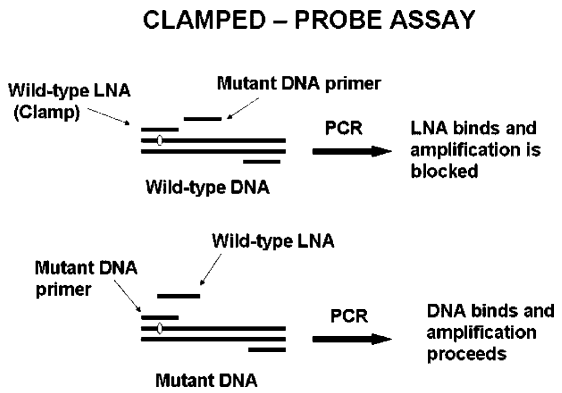


Figure 16. Schematic representation of Clamped-probe assay. The non-extendable LNA (phosphorylated at 3′-end) clamp binds preferentially to the wild-type DNA sequence, thus, suppressing its PCR amplification and changing the PCR bias toward the mutated target. Stringent PCR conditions allow annealing and extension of mutated primer only.

selectivity, such as, for example, a molecular beacon (MB). Molecular beacons are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure (Figure 17). The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore is covalently linked to the end of one arm, and a quencher is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing a target sequence, they undergo a conformational change that enables them to fluoresce brightly. Because of their high signal/background ratio and their high selectivity in molecular recognition, MBs have found broad application in *in vitro* assays. Lately, LNAs have been compared to the analogous DNA-MB molecular beacons consisting of a 19-mer loop and a 6-mer stem, and LNA-MBs proved to be far superior to DNA-MBs. The LNA-MBs were highly thermostable and did not open even at 95 °C. The presence of the target strand, however, leads to their efficient opening and subsequent hybridization even at room temperature without loss of higher selectivity, nuclease stability, or signal-to-background ratio in the intracellular environment. The longer lifetime with extremely low background of LNA-MBs makes them an excellent probe for gene expression in the cellular studies.[129]

With LNA-substituted oligonucleotides outperforming DNA probes, it is desirable to develop general software tools to provide optimal designs of LNA-modified oligonucleotides for application in functional genomics. Tolstrup et al. developed a new computer program, OligoDesign,[130] which ranks a probe on the basis of a number of parameters for application in microarrays. They have used this program successfully to design a *Caenorhabditis elegans* LNA-oligonucleotide microarray to monitor expression of a set of 120 potential marker genes for stress and toxicological processes and pathways.[131] The design is based on several parameters including recognition and filtering of the target sequence in the genome of interest; genome-wide BLAST analysis using blastn[132] for minimized cross-hybridization; LNA melting temperature prediction using thermodynamically derived formula,[132,133] modified to predict melting temperature if LNA/DNA mixmers based on melting temperature measurements of >1400 DNA−LNA duplexes; prediction of LNA self-annealing using the Smith-Waterman sequence alignment algorithm;[134] and finally, secondary structure prediction using the Nussinov algorithm[135] for the LNA probe as well as the target nucleotide sequence. Individual scores for the aforementioned parameters are calculated for each possible probe in the query gene and run through a fuzzification process. The OligoDesign program is freely accessible at http://lnatools.com.

3.2.2. RNA Capture Probes

In situ detection by hybridization of RNA with complementary probes is a powerful technique for studying expression level and localization of RNA within all types of eukaryotic cells ranging from yeast to humans as well as for probing tissue sections or whole-mount preparations. The rapid development of LNA-modified oligonucleotide probes has significantly improved the sensitivity and specificity of RNA detection. Our own data,[136] based on FRET-binding study and electrophoretic mobility shift assay, highlighted the superiority of LNA-based oligonucleotide to target highly structured nucleic acid targets, which are otherwise not accessible to the conventionally used unmodified oligonucleotides. The high affinity and high sensitivity of LNA-based probes indicated that they can be even employed in cases where detection of target is limited by its smaller size, lower abundance, or structural features.[136] The attractive set of properties offered by LNA has been exploited in efficient isolation of intact poly $(A)^+$ RNA from guanidine thiocyanate lysed *C. elegans* worm extracts as well as from lysed human K562 and vincristine-resistant (K562/VCR) leukemia cells. When every second thymidine in these probes was substituted with LNA-T, mRNA yield increased by 30−50-fold.[137] Further, the probes were highly efficient in isolation of poly $(A)^+$ RNA from extracted total RNA samples in low salt binding buffers. Thus, the LNA oligo (T) method could be automated for streamlined high-throughput expression profiling by real-time PCR after covalently coupling the LNA affinity probe with a solid surface such as a microtiter plate or a magnetic particle.[137]

3.2.3. miRNA Detection

One of the most promising applications of LNAs is their use in efficient detection and characterization of microRNAs (miRNAs), which are small (20−23 nucleotide) noncoding

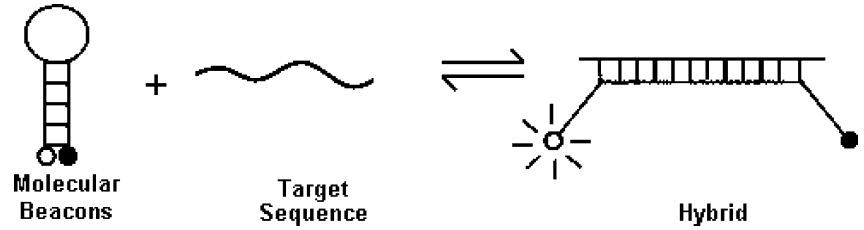


Figure 17. Schematic representation of the operation of molecular beacons.

RNAs that regulate gene expression by base-pairing to partially complementary mRNAs. Regulation by miRNAs is important in embryonic development. However, the detection and characterization of miRNAs have been technically challenging because of their small size. DNA oligonucleotides with several positions substituted by LNA residues have been used in Northern blot analysis to significantly increase detection of low-abundance miRNAs by at least 1 order of magnitude.[138] Besides being highly efficient as Northern blot probes, LNA-modified oligonucleotides have been used for *in situ* hybridization (FISH) both on fixed yeast cells to detect specific mRNAs[139] and on whole-mount zebrafish embryos to detect the temporal and spatial expression pattern of 115 conserved vertebrate miRNAs.[140] To explore the usefulness of LNA-modified probes in the detection of miRNAs in other organisms, the conditions for hybridization of LNA probes to miRNAs have been optimized. Subsequently, numerous previously unknown miRNAs were identified in mouse and frog embryos using *in situ* hybridization.[141] Single molecular LNA probes have allowed quantification of miRNAs in humans.[142] The technique involved using two spectrally distinguishable fluorescent locked nucleic acid probes for detection of the miRNA of interest and then directly counting the tagged molecules on a single molecule detection instrument. The high sensitivity of this technique allowed the detection and quantification of expression at low concentration in 16 different tissues of 45 human miRNAs and yielded a quantitative differential expression profile that correlated well with published reports.[142] Recently, LNA probes have been used in microarray-based miRNA profiling assays that allow parallel screening for the expression of a large number of miRNAs.[143] This novel assay (miChip) exploits the biophysical properties of LNA to design capture probes with a uniformly normalized T_m, which in turn allows establishing uniform, high-affinity hybridization conditions suitable for all miRNAs. Hybridization conditions optimized for high-stringency binding not only accurately detect the expression of miRNAs, but also increase the specificity of detection for related miRNA family members that differ by a single-nucleotide.[143] Another miRNA expression profiling assay utilized the RAKE (RNA-primed, array-based, Klenow Enzyme) miRNA-microarray platform in conjunction with LNA-based *in situ* hybridization (LNA-ISH) to analyze coordinated miRNA expression from the tissue level to subcellular levels. The RAKE microarray, which involves on-slide application of Klenow fragment of DNA polymerase to extend unmodified miRNAs hybridized to immobilized capture probes, revealed tissue-specific expression of particular miRNAs. Subsequently, LNA-based *in situ* hybridization was applied to refine the data obtained by RAKE and to localize the cellular and subcellular distribution of miRNAs. This allowed differential spatial expression profiling of miRNA.[144]

The use of LNA probes is no longer limited to the detection or expression profiling of miRNAs, but extends even to the understanding of the precise molecular functions of specific miRNAs. In this application, the same LNA probes are used as 'antagomirs' to silence the functioning of endogenous miRNAs. These LNA-antagomirs are short RNA molecules that bind to miRNA and sequester it in a duplex form so that it is no longer available to bind to its target mRNA.[145] These loss-of-function studies allow the characterization of specific functions of particular miRNAs and, thus, the identification of miRNA-regulated genes which are thereby, marked as potential new therapeutic targets. LNA-mediated inhibition of miRNA function has been studied in cultured cells with the use of LNA antagomirs to inhibit the well-characterized interaction between *Drosophila melanogaster* bantam miRNA and its target gene *hid*. The *bantam* miRNA controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. LNA-modified anti-miRNA oligonucleotide (antagomir) could mediate specific inhibition of both endogenous miRNAs and exogenously applied miRNAs, resulting in subsequent derepression of cognate target protein expression.[146] LNA-based loss-of-function studies have also been used to establish the role and biological significance of elevated levels of miR-21 in glioblastoma, a highly malignant human brain tumor.[147] Suppression of miR-21 by LNA-modified oligonucleotides (22-mer) led to a drastic reduction in the number of transfected cells. Further, studies showed that the decrease in cell number was associated with a marked increase in caspase activity that subsequently acelerated the rate of cell apoptosis in antisense treated cells. The study thus established miR-21 as an antiapoptotic factor that may function as a micro-oncogene in glioblastomas by blocking expression of key apoptosis-enabling genes.[147] In another study, LNA/DNA mixmers were used to inhibit the functional activity of endogenous miR-181, thus, preventing its interaction with its target mRNA, Hox-A11, which is an essential regulator of embryonic uterine development and the cyclic development of the adult uterine endometrium.[148] Inhibition in this case was sequence-specific, with there being no cross-inhibition between different miRNAs, and with the presence of four mismatches abolishing the inhibition. When transfected into the cells, the LNA/DNA mixmers were able to induce complete disappearance of their target miRNA, as observed by Northern blot analysis. This is attributed to the formation of highly stable LNA/DNA mixmer•miRNA duplexes inside the cells that are subsequently able to resist the denaturing conditions used for Northern blot, thereby preventing miRNA detection. Significantly, inside the cells, LNA/DNA mixmer affected not only the mature miRNA, but also the apparent levels of its respective 'precursor' miRNAs, which suggests that LNA might affect the regulatory loop in miRNA processing, thereby influencing the actual levels of miRNA precursors.[148]

3.3. Other Uses of LNAs

Successful design specification[149,150] is one of several distinct advantages of LNA primers over DNA controls. These attributes allow highly sensitive and highly specific amplification of PCR targets of LNA primers. LNA may also be used as an inhibitor in PCR, or in real-time PCR. Real-time PCR is a highly sensitive method for the detection of low-abundance mRNA. However, one of the most serious drawbacks of this method involves possible amplification of false positives, which can interfere with mRNA quantification, due to DNA contamination in the RNA samples. With the use of an LNA oligonucleotide complementary to the intron of a gene, genomic DNA amplification could be eliminated without affecting the amplification of reverse-transcribed spliced mRNA. When this strategy was used for the B-cell plasma cell marker, x-box binding protein 1 (XBP1) gene, genomic XBP1 amplification was blocked for both PCR and real-time PCR but not for cDNA XBP1.[151] These studies thus predicted a promising role for LNA in

enhancing assay specificity by eliminating false-positive genomic amplification and a particular usefulness in amplifications dealing with low-abundance mRNA.

The application of LNA in "gene repair" or "targeted nucleotide exchange technique" is currently being explored.[152] LNA successfully directs single-base-mutation repair in *Sacchromyces cerevisiae*. Here, the correction of point and frameshift mutations of the mutant hygromycine-resistant gene was facilitated by a 24-mer oligonucleotide vector containing LNA residue at each terminus, which serves to target either strand of the gene while hybridizing to the site with the mismatched base pair. The nucleotide exchange event was then catalyzed by proteins involved in DNA recombination and repair pathways.[152] The potential of LNAs for gene editing has been validated in higher eukaryotes by directing mutagenesis at two different sites, the *eBFP* (blue fluorescence protein) and the *rd1* mutant retinoic photoreceptor βPDE (rod photoreceptor-specific phosphodiesterase gene).[153] Again, the use of a short 25-mer oligonucleotide with flanking LNAs exhibited efficient dose-dependent mutagenesis and also allowed stable transmission of the modification during cell division. The latter is a feature important for reliable cloning of the modified cells. Finally, the survival of photoreceptors in the retinas of *rd1* mutant mice was promoted by such oligonucleotides, which suggested the possibility of their successful use *in vivo*. We, thus, speculate that LNAs can act as powerful reagents for targeted mutagenesis in cultured cells and also in animal models of human disease.

LNA oligonucleotides can efficiently attach fluorophores as functional moieties to super-coiled plasmid DNA in a sequence-specific manner. LNA oligonucleotides bind to plasmid DNA by strand displacement far more stably than peptide nucleic acids (PNA) and, thus, allow particle-mediated delivery of many functional peptides or adjuvant moieties without interfering with the gene expression.[154] In addition, they have also been used to facilitate attachment of functional moieties by PNA oligomers. Here, the LNA oligomers act as openers by hybridizing to the partially overlapping sites of the opposite plasmid DNA strand and facilitating strand invasion by bisPNA (oligomers with two identical peptide nucleic acid sequences joined by a flexible hairpin linker) anchor peptides (Figure 18). In this way,

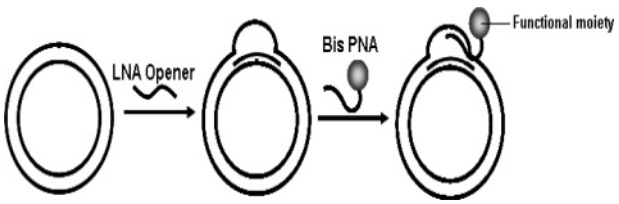


Figure 18. Schematic representation of attachment of functional moiety (peptide) by bisPNA anchor molecules by strand displacement, facilitated by LNA-modified openers.

greater than 80% hybridization is obtained at PNA-to-plasmid ratios as low as 2:1. The high biological stability of the LNA openers in combination with bis PNAs as anchor molecules thus offers an attractive strategy to couple different functional moieties to the plasmid in a sequence-specific manner.[155]

The successful use of LNA technology has allowed application of its functionalized derivatives for numerous other versatile applications as well. The pyrene-functionalized 2′-amino-derivative of LNA is increasingly being adopted for nucleic acid detection in homogeneous fluorescence

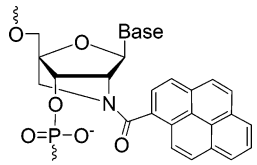


Figure 19. 2′-*N*-(Pyrene-1-yl)carbonyl-2′-amino-LNA monomer.

assays (Figure 19).[156,157] Typically, molecular beacons relying on distance-dependent fluorescence quenching are used in such assays. However, such probes often form secondary structure and generate false-positive signals. Therefore, an alternative strategy is realized by tethering reporter groups to single-stranded oligonucleotides and observing modulation of hybridization-induced fluorescence intensity. 2′-*N*-(Pyren-1-yl)carbonyl-2′-amino-LNA is promising in this context, since it displays both a large increase in thermal stability against DNA or RNA with excellent mismatch discrimination and a large hybridization-induced increase in fluorescence intensity, upon duplex formation. Upon hybridization to DNA or RNA, the dioxabicyclo[2.2.1]heptane skeleton and amide linkage of the modifucation fix the orientation of the pyrene moiety in the minor groove of the nucleic acid duplex. Pyrene and nucleobase interactions, which typically quench fluorescence, are thereby reduced, and hybridization-induced fluorescence intensity measured at $\lambda_{em} = 383$ nm is increased.[156] The detection of nucleic acid is thus facilitated in the presence of such multilabeled pyrene-functionalized 2′-amino-LNA-based oligonucleotides.

LNAs have been used to improve the biostability and specificity of the Tenascin-C binding 39-mer oligonucleotide aptamer TTA1, which structurally recognizes human Tenascin-C (TN-C), a hexameric protein found in the extracellular matrix involved in tumorigenesis, embryogenesis, and wound healing. On the basis of this feature, TTA1 labeled with technetium-99m (Tc-99m) is a promising candidate for imaging tumors expressing TN-C.[157] The TTA1 aptamer currently in clinical trials has a three-stem junction where the binding domain is centered. In this case, the stems play an important role in the binding and maintenance of the overall structure. Experiments with different oligonucleotides (unmodified RNA, 2′-*O*-methyl RNA, 2′-fluoro RNA, and LNA) have shown that incorporation of LNA and 2′-*O*-Me modifications in the non-binding double-stranded stem 1 confer desirable properties to the aptamer; the LNA-modified TTA1 derivative exhibited significant stem stabilization and markedly improved plasma stability while maintaining its binding affinity to the target. In addition, it was found that the presence of LNA leads to a higher tumor cell uptake and longer blood retention.[158]

4. Conclusions and Future Prospects

With an emphasis on improving stability and specificity, nucleic acid analogues are finding numerous applications in contemporary molecular biology. LNA is one such analogue that has recently gained much attention for its favorable properties. The stability of LNA-modified oligonucleotides in biological fluids, their lack of toxicity, and their improved hybridization behavior have made them promising therapeutic tools for use in antisense and antigene applications involved in the therapy of many pathophysiological conditions including cancer. High-affinity LNAs can be readily designed as fully modified LNA, LNA/DNA chimera, LNA/RNA chimera, or combined with other modifications such

as phosphorothioate linkages or 2′-*O*-Me-RNA. This makes LNA compatible with different oligonucleotide chemistries and technologies. Further, the flexibility in the architecture of LNA oligonucleotides can be well-tuned to achieve the desired level of gene expression, for example, RNase H-mediated gene silencing by employing LNA gapmers, or LNA chimeras-mediated increase in protein expression by blockade of splice sites.

In addition to creating avenues for gene-based therapy, the heightened ability of LNA to discriminate between matched and mismatched target nucleic acids has increasingly attracted the attention of the genotyping community. Since hybridization-based genotyping methodologies are limited by the presence of thermally stable mismatches, the inclusion of LNA residues in probe oligonucleotides significantly improves their differentiation characteristics, thus, allowing increasing use of LNA-based primers and probes in genotyping and diagnosis. In comparison with a DNA probe, the increased ΔT_m of an LNA probe between/against the perfectly matched and mismatched targets broadens the interval required to set optimal conditions for specific hybridization to a matched target.

A possible application of LNA probes is in the field of pharmacogenomics, which requires a robust, reliable, and cost-effective method for microarray-based gene expression profiling of individual patients. At present, the potential for a small set of 8- and 9-mer probes to identify every gene is being investigated and should greatly simplify the probe set.[159] Another breakthrough in the technology of LNAs is their use in the discovery and determination of the respective functions of miRNAs. Understanding the role of microRNAs in the fundamental processes associated with complex diseases such as asthma, chronic obstructive pulmonary disease, cancer, chronic infections, and immune disorders may aid in disease diagnosis and prognosis and potentially identify new therapeutic targets. The use of LNA-antagomirs to target specific miRNAs *in vivo* represents an important therapeutic strategy for silencing miRNAs in disease conditions.

Numerous other versatile applications of LNAs are foreseen: The high binding affinity of LNA combined with retention of sequence specificity can be exploited to trap a number of biologically relevant secondary structures formed in both RNA and DNA, so that, for instance, short LNA-modified oligonucleotide stretches can be used to trap intramolecularly formed G-rich quadruplex motifs which are formed transiently in a number of cellular events such as replication, recombination, transcription, and telomere elongation, and are therefore thought to play important roles in a number of biological processes. Targeting of these structures by high-affinity LNA-modified ODNs will promote Watson and Crick base pairing over quadruplex formation and will therefore have a profound effect in better understanding and modulation of quadruplex-mediated biological processes. Quadruplex-regulated gene expression can also be modulated by use of G-rich LNA-modified sequences,[160] which are capable of forming quaduplex structures. Such multistranded LNA oligonucleotides can be used as aptamers to target transciption-activating proteins that normally interact with G-quadruplex structures to regulate a number of biological processes. This strategy thus allows the biological application of LNAs as tools to design aptamer oligonucleotides.

A novel application of LNA might involve the use of LNA-modified ATPs or GTPs, which will allow high-affinity binding of these NTPs toward their target ATP or GTP binding proteins (such as kinases or GPCRs), with the potential use of monitoring effects of their prolonged expression or activation. LNA can also be used to add functionality to a wide range of DNA constructs. We propose that several peptides, enzymes, drugs, and adjuvant moieties can be coupled with DNA in a controlled manner by attaching functional moieties to the 3′- and 5′-ends of LNAs. This approach may greatly improve gene therapy and DNA vaccines by increasing the potency and efficiency of the therapeutic agent and gene delivery systems.

Many applications of LNA rely on the use of N-alkylated derivatives of LNA, such as attachment of fluorophores to the N-2′-position of 2′-amino-LNA via short amide linkages, which may become a general paradigm for tethering of fluorophores to oligonucleotides without the typical loss in fluorescence intensity.[157] Because of their resistance to RNase H and 3′-exo- and endonucleases, pyrene-functionalized 2′-amino-LNA probes could, in this context, significantly improve real-time *in vivo* imaging of RNAs. In addition, functionalization of 2′-amino-LNA with the pyrene moiety can be used to couple oligonucleotides with carbon nanotubes (CNT). Carbon nanotubes find wide use in nanobiotechnology in areas ranging from molecular electronics to ultrasensitive biosensors, but successful applications still await the development of efficient methods for enhancing their water solubility. The high affinity of pyrene toward carbon nanotubes[161] is, in this case, of great use, since pyrene attached to LNA may serve as a linker to tether LNA-modified oligonucleotides to the surface of carbon nanotubes. Grafting of oligonucleotides not only increases the solubility and biocompatibility of carbon nanotubes, but also provides them with specific handles to make them suitable for several applications.

Substantial effort is also currently being applied to the utilization of LNA•PNA hybrids and to the programmed assembly of nanoscale devices.[162] We suggest that LNA•PNA hybrids may serve as more stable templates for organizing nanoparticles and nanowires, quantum dots, carbon nanotubes, dendrimers, micron-sized polystyrene beads, and virus particles, and for attaching nano- and micron-sized particles to substrates, thereby enhancing their physical stability without the loss of chemical reactivity. In each of the instances cited, nucleic acids act as templates to place reactants in close proximity on hybridization, thereby facilitating chemical reactions. The stability of a LNA•PNA duplex is higher than the stability of individual LNA•DNA or PNA•DNA. Also, stability equivalent to a DNA•DNA duplex can be achieved with a LNA•PNA duplex of a significantly shorter length.[162] The LNA•PNA combination, thus, is a unique example of an orthogonal programmable assembler that can serve quite well as a replacement for DNA in programmable assembly.

LNA is, thus, likely to make a strong impact on every sphere of biotechnology and medicine. However, a thorough understanding of the biophysical and molecular aspects of the hybridization thermodynamics of LNA is necessary for facilitating its proper design and use in diverse applications. This will not only enable us to tune its architecture according to the functional requirements, but also allow us to predict and design further analogues with better properties and higher efficiency.

5. Abbreviations

LNA/DNA	single-strand-containing LNA and DNA nucleotides
LNA•DNA	double-stranded hybrid containg an LNA-modified and a DNA strand
PS	phosphorothioates
2′-*O*-Me	2′-*O*-methyl RNA
FANA	2′-deoxy, 2′-flouro-β-D-arabino nucleic acid
PNA	peptide nucleic acids
siRNA	small interfering RNA
RISC	RNA-induced silencing complex
AS	antisense
AON	antisense oligonucleotide
CD	circular dichroism
NMR	nuclear magnetic resonance
T_m	temperature of melting
TFO	triplex-forming oligonucleotide
PCR	polymerase chain reaction
NF-κB	necrosis factor- κB
Pfu	*Pyrococcus furiosus* derived DNA polymerase
Bal31	*Alteromonas espejiana* derived endonuclease
CV-1	cells derived from kidney of an adult male African green monkey (***Cercopithecus aethiops***)
HeLa	Henrietta Lack cervical cancer cells
MCF-7	epithelial breast cancer derived cell line
UTR	untranslated region
SNP	single nucleotide polymorphism
ICAM	intracellular adhesion molecule
FISH	fluorescence in situ hybridization
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1

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