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Locked nucleic acids: a family of high affinity nucleic acid probes

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Abstract

The structure and synthesis of LNA (locked nucleic acid) is presented. LNA is defined as an oligonucleotide containing one or more 2′-O, 4′-C-methylene-β-D-ribofuranosyl nucleotide monomer(s). The improved synthesis of the thymine LNA monomer is shown. The LNA molecular family is presented and the member classes are described in relation to their chemical structures. The hybridization property of each member is shown and similarities and differences in the properties of the members are discussed. Hybridization kinetics of oxy-LNA oligonucleotides is shown and it is concluded that the hybridization rate is nearly diffusion controlled. Finally, the perspective of the broad biological application of LNA is discussed.

1. Introduction

Natural dsDNA exists at physiological pH as a B-form helix, whereas dsRNA exists as an A-form helix. This morphological difference originates in the difference in the preferred sugar conformations of the deoxyriboses and the riboses. The furanose ring of deoxyribose exists at room temperature in an equilibrium between C2′-*endo* (S-type) and C3′-*endo* (N-type) conformation with an energy barrier of ∼2 kcal mol^{-1} (figure 1) [1–3]. The C2′-*endo* (S-type) conformation gives rise to the B-form helix whereas the C3′-*endo* (N-type) conformation gives rise to the A-form helix.

For deoxyribose the S-type conformation is slightly lowered in energy (∼0.6 kcal mol^{-1}) compared to the N-type and this explains why DNA is found in the S-type conformation. For ribose the preference is for the N-type and thus RNA adopts the A-form helix. The A-form helix is associated with higher hybridization stability.

It is well known that a tremendous effort has been devoted to the preparation of DNA analogues with better binding properties to nucleic acid than the native counterparts. The most important guideline in this work has been to design the DNA analogues in such a way that the analogue would attain the N-type/'RNA'-like conformation in order to obtain the higher affinity [4].

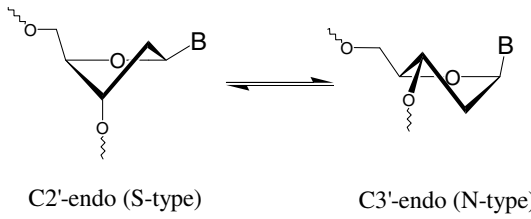

Figure 1. Furanose conformations.

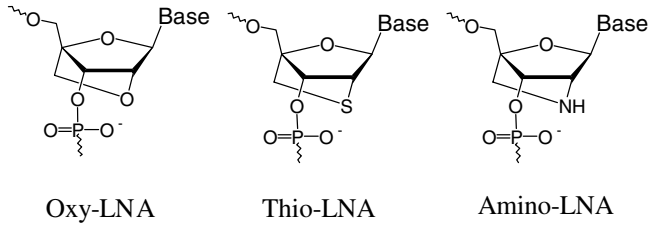

Figure 2. Chemical structure of the oxy-LNA and the thio-/amino-LNA analogues.

In 1997 Obika *et al* [5] disclosed the synthesis of the 2′-O–CH_2-4′ bicyclic nucleoside in which the conformation due to the bicyclic structure is locked in the N-conformation, and in the same year Wengel *et al* [6, 7] prepared the first LNA (locked nucleic acid) and showed the unprecedented affinity of LNA towards nucleic acids (figure 2). It turned out the that LNA became the single nucleic acid modification that contributes to the highest affinity ever obtained by Watson–Crick hydrogen bonding, and most importantly, the increased affinity came together with very high sequence specificity for the fully matched nucleic acid target. The first LNA monomer was based on the 2′-O–CH_2-4′ bicyclic structure which is now called the oxy-LNA (figure 2) [8, 9].

The 2′, 4′ bicyclic structure brings not only unprecedented affinity/specificity to fully modified LNA oligonucleotides, but LNA monomers can be mixed and act co-operatively with DNA and RNA monomers in chimeric oligonucleotides. Interestingly this co-operative binding of LNA was also found in combination with most of the known nucleic acid analogues such as phosphothioates and 2′-O-alkyl modified RNA [4, 10].

Right after the discovery of oxy-LNA the bicyclic furanosidic structure was chemically modified. Thus, the 2′-S–CH_2-4′ (thio-LNA) and the 2′-NH–CH_2-4′ (amino-LNA) bicyclic analogues were prepared [11, 12] (figure 2).

The parent oxy-LNA monomer and the thio-/amino-LNA analogues are formed by the 2′-X–CH_2-4′ bicyclic furanose. Due to the configuration of these LNA structures they are more specifically termed β-D-oxy-LNA and β-D-thio/amino-LNA. Beta (β) relates to the fact that the nucleobase and the 5′-OH are on the same side of the furanose; D relates to the fact that the furanose is configured in the D-series [13].

If the chemical constitution is kept constant in the bicyclic structure of LNA the structure can be chemically changed to form a series of closely related molecules, diastereoisomers, in which the stereochemistries at one or more chiral centres, but not all, are changed. A series of LNA diastereoisomers has been prepared [14–21] (figure 3).

In the strict definition LNA is understood as an oligonucleotide containing one or more 2′-O, 4′-C-methylene-β-D-ribofuranosyl nucleotide monomer(s). As it appears from the above the monomer universe is easily chemically expanded to comprise LNA analogues

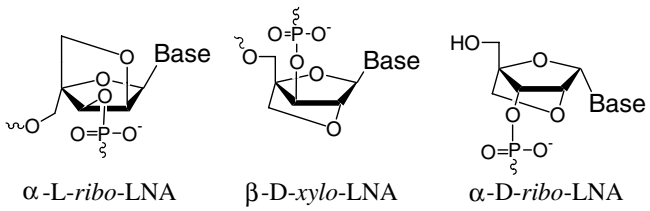

Figure 3. Chemical structure of LNA diastereoisomers.

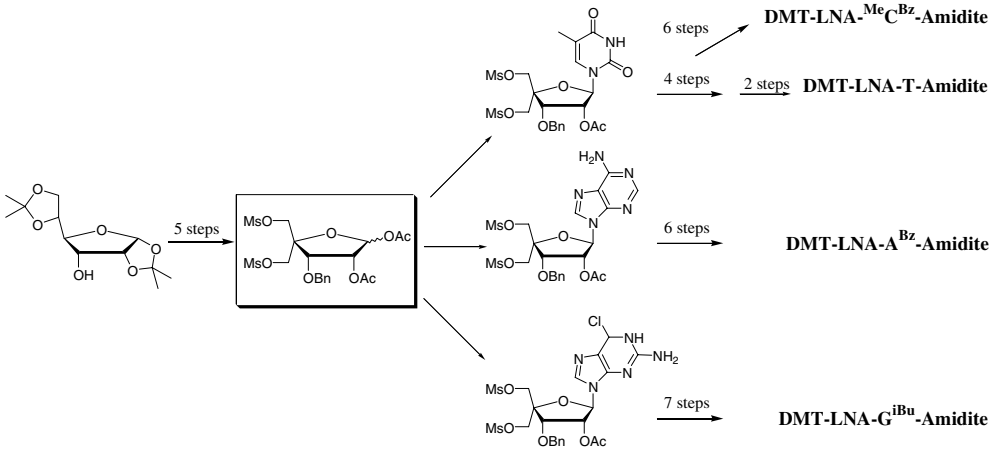

Figure 4. Flow scheme showing the convergent synthesis of LNA amidites.

(thio-/amino-LNA). Furthermore, the configuration of the original oxy-LNA structure can be changed to form a group of diastereoisomers of LNA that also are easily incorporated into DNA and RNA. Therefore, in the broader understanding LNA and the closely structural 'relatives' represent a family of compounds of which nearly all of them contribute to higher affinity when incorporated into oligonucleotides. Typically the increase in T_m per modification varies from +5 to +11 °C. The highest T_m increase is found for LNA oligonucleotides targeting RNA and no other DNA analogues have reproducibly shown such high affinity for nucleic acids.

1.1. Synthesis

The oxy-LNA monomer synthesis has been made from two strategies: 1, the nucleoside route [5, 22], and 2, the sugar route.

In the nucleoside route the LNA nucleosides are made from the corresponding RNA nucleosides, whereas in the sugar route a common sugar intermediate is used for all four monomers [6, 7] (figure 4). The latter convergent strategy has shown to be a very efficient in producing the four monomers and it is also useful for large-scale production of the LNA amidites. I will focus here on this strategy.

The starting material is the commercially available 1, 2:5, 6-di-*O*-isopropylidene-α-D-allofuranose. Protection of the 3′-OH is done by alkylation with benzylbromide (figure 5). The 5,6-isopropylidene group can be selectively removed in diluted acetic acid. Oxidation of the diol with periodate followed by aldol condensation and Cannisaro reaction yields the 4′-*C*-hydroxymethyl furanose ***X*** (figure 5). Dimesylation of the 4′-*C*-hydroxymethyl furanose

Table 1. Hybridization studies with 9-mer LNAs towards complementary DNA and RNA. A = nucleotide monomer with an adenin-9-yl base, C = nucleotide monomer with a cytosin-1-yl base, G = nucleotide monomer with a guanin-9-yl base, T = nucleotide monomer with a thymin-1-yl base. ^{Me}C = nucleotide monomer with a 5-methylcytosin-1-yl base. Oligo-2′-deoxyribonucleotide sequences are depicted as d(sequence), oligoribonucleotide sequences as r(sequence) and 2′-O-Me-oligoribonucleotide residues are underlined. LNA monomers are shown in boldface with superscript 'L'. Ps designates phosphothioates and subscript 'S' denotes a phosphorothioate linkage.

Entry	Sequence	DNA target T_m (°C)	RNA target T_m (°C)
1 Reference DNA	5′-d(GTGATATGC)	28	28
2 Oxy-LNA/DNA chimera	5′-d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	44	50
3 Oxy-LNA/RNA chimera	5′-r(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	55	63
4 Fully modified oxy-LNA	5′-($\mathbf{G^LT^LG^LA^LT^LA^LT^LG^{LMe}C^L}$)	64	74
5 Oxy-LNA/2′-OMe-RNA chimera	5′-(GTGA$\mathbf{T^L}$ATGC)	32	43
6 Oxy-LNA/2′-OMe-RNA chimera	5′-(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	49	59
7 Ps-oxy-LNA/-DNA chimera	5′-d($G_S\mathbf{T^L_S}G_SA_S\mathbf{T^L_S}A_S\mathbf{T^L_S}G_SC$)	41	47

$\boldsymbol{XX}$ followed by a one-pot acetolysis/acetylation procedure forms the common coupling intermediate $\boldsymbol{Y}$. The synthesis of the LNA nucleosides follows principally the same route but I will in this context only describe the synthesis of the thymine nucleoside and refer the interested reader to the excellent chemistry papers [8, 9, 23, 24]. Thymine is coupled to the intermediate $\boldsymbol{Y}$ by stereoselectively standard Vorbrüggen conditions and subsequent base induced cyclization affords the protected 2′-*O*, 4′-*C*-methylene-β-D-ribofuranosyl nucleosides. The 5′-*O*-mesylate is displaced by benzoate that is subsequently hydrolysed and final debenzylation of the 3′-*O*-Bn protected alcohol yields the LNA-thymine nucleoside. The 5′-OH Dmt protected nucleosides are phosphitylated using 2-cyanoethyl-N, N, N′, N′-tetraisopropylphosphorodiamidite and DCI, a procedure that provides the LNA amidites in yields higher than 90% even after column purification [25].

The overall yield of the LNA amidites is in the range of 35–45% depending on which monomer is prepared. This is an impressive yield considering the multi-step nature of the synthesis (figure 4).

Oligomerization of the amidites is performed on standard DNA synthesizers and follows in general standard DNA synthesis procedures. The only significant changes are that the coupling reaction is slightly prolonged (300 s) and that the oxidation is also prolonged (50 s). The work-up of the oligonucleotides follows standard procedures [26].

1.2. Thermal denaturation of oxy-LNA heteroduplexes

One of the intriguing properties of LNA is that the monomer can be incorporated in regular DNA and RNA and that the structure can be adapted within a regular DNA/RNA oligonucleotide in such a way that each incorporation of an LNA monomer adds co-operatively to the binding of the oligonucleotide to a complementary DNA/RNA. In this context the binding of LNA containing oligonucleotides have been thoroughly examined. In table 1 are shown melting temperatures (T_m) of a 9-mer mixed LNA sequence with different constructs toward complementary single-stranded DNA and RNA. The values of the corresponding unmodified DNA and RNA duplexes are shown in entry 1 [6–9, 27, 28].

The data in table 1 clearly show that oxy-LNA binds with higher affinity to nucleic acids. Entries 2–4 illustrate that increasing the number of LNAs increases the T_m no matter

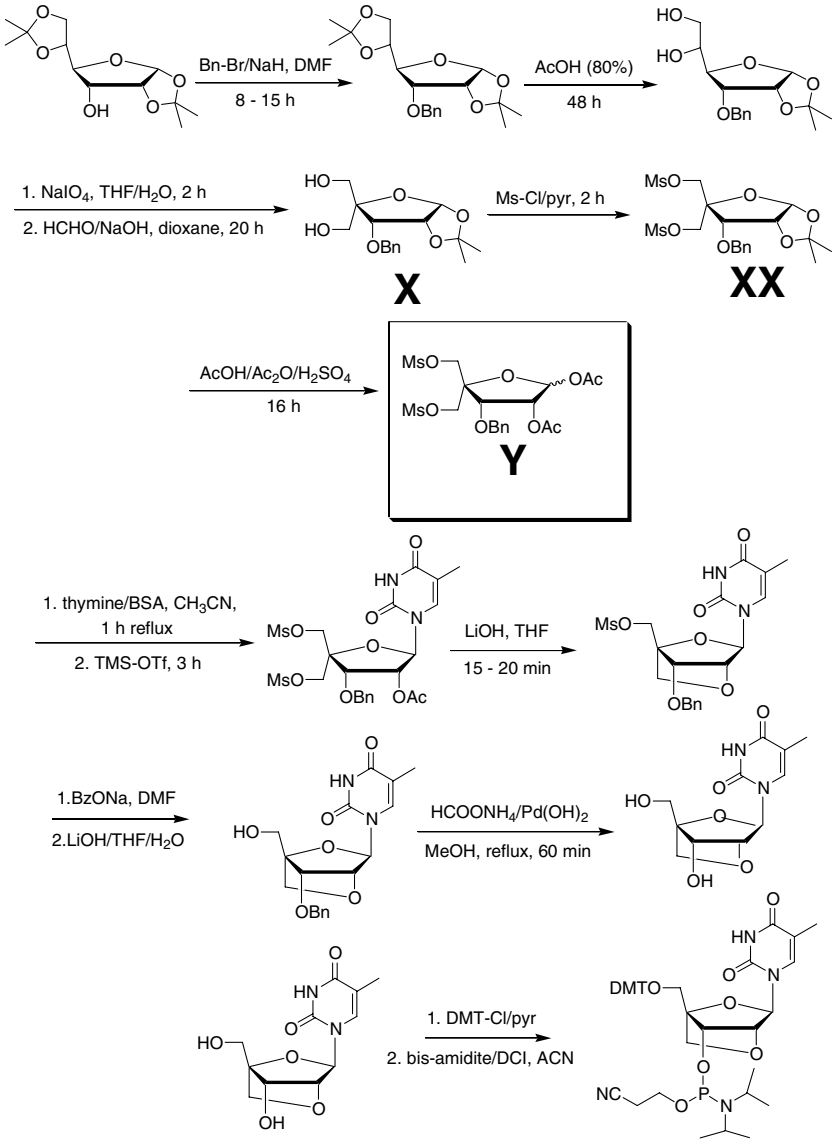

Figure 5. LNA T-monomer synthesis.

whether the oxy-LNA is incorporated in DNA or in RNA. Entries 5 and 6 shows that LNA can be incorporated in more complex designed chimerae, thus a chimera containing DNA and the 2′-OMe modification of DNA can also accommodate oxy-LNA. In this 9-mer oligonucleotide three different residues, DNA, 2′-OMe and LNA, are incorporated and even in this construct oxy-LNA acts co-operatively and every oxy-LNA residue adds additively to the affinity. Entry 7 shows that oxy-LNA can be incorporated in oligos containing other backbones than the regular phosphodiester backbone. In this case oxy-LNA residues are incorporated between phosphothioate inter-nucleoside linkages. This backbone modification is used in many biological applications due to its larger bio-stability as compared to the native phosphodiester backbone. It is shown in the table (entry 7) that three oxy-LNA thymines

Table 2. Hybridization studies with 9-mer LNAs towards complementary DNA and RNA. LNA monomers are shown in boldface with superscript 'L'. A = nucleotide monomer with an adenin-9-yl base, C = nucleotide monomer with a cytosin-1-yl base, G = nucleotide monomer with a guanin-9-yl base, U = nucleotide monomer with a uracil-1-yl base, T = nucleotide monomer with a thymin-1-yl base. Oligo-2′-deoxyribonucleotide are called d(sequence). Superscript 'LS' is a thio-LNA monomer, superscript 'LNH' an amino-LNA monomer and superscript 'LNR' a methylamino-LNA monomer.

Entry	Sequence	DNA target T_m (°C)	RNA target T_m (°C)
1 Reference DNA	5′-d(GTGATATGC)	28	28
2 Oxy-LNA/DNA chimera	5′-d(G$\mathbf{T^L}$GA$\mathbf{T^L}$A$\mathbf{T^L}$GC)	44	50
3 Thio-LNA/DNA chimera	5′-d(G$\mathbf{U^{LS}}$GA$\mathbf{U^{LS}}$A$\mathbf{U^{LS}}$GC)	42	52
4 Amino-LNA/DNA chimera	5′-d(G$\mathbf{T^{LNH}}$GA$\mathbf{T^{LNH}}$A$\mathbf{T^{LNH}}$GC)	39	47
5 Methylmino-LNA/DNA chimera	5′-d(G$\mathbf{T^{LNR}}$GA$\mathbf{T^{LNR}}$A$\mathbf{T^{LNR}}$GC)	39	49

increase the melting temperature almost 30 °C corresponding to +10 °C per modification. It is in this context important to note that the specificity of the hybridization has been thoroughly examined against single base pair mis-matches. The discrimination of oxy-LNA against mis-matches is at least as high as for the corresponding DNA residues and it is for a majority of cases significantly higher measured as ΔT_m values in degree celsius.

1.3. Thermal denaturation of LNA analogue heteroduplexes

As mentioned above the oxy-LNA molecule has been chemically derivatized. The most obvious modifications were to change the heteroatom in the 2′-O–CH_2-4′ bicyclic structure. The corresponding LNA analogues, thio-/amino-LNA, were prepared and their hybridization characteristics were examined [11, 12, 29].

Not surprisingly the T_m values of the thio-LNA analogue closely resembled the values of the oxy-LNA (table 2), but it was interestingly to note that the amino-LNA also had increased affinity despite the fact that the amino functionality will create a positive charge on the bicyclic furanose moiety. This is probably part of the explanation of the moderate reduction in the affinity of the amino-LNA compared to oxy-/thio-LNA (table 2, entry 4).

These data illustrate that the 2′-X–CH_2-4′ bicyclic structure in the β-D configuration is a more general high affinity structure and that the introduction of other hetero-atoms than oxygen do not destroy the high affinity nucleic acid binding. The amino-LNA and methylamino-LNA add a different functionality to LNA. Thus, the amine is positively charged at physiological pH and the amine can be used to further functionalize LNA analogues because the amine may serve as an inter-linking group between the LNA and other ligands. When properties other than hybridization are considered it is reasonable to assume that the heteroatoms of the LNA analogues will induce differences in the physical and chemical properties compared to the oxy-LNA. This differentiation in overall properties of the oxy-LNA and the LNA analogues adds an interesting perspective to the LNA family of compounds.

2. Thermal denaturation of LNA diastereoisomer heteroduplexes

α-L-LNA is one of the most studied LNA diastereoisomers (called α-L-*ribo*-LNA in figure 3) and the molecule exhibits many interesting properties that warrant many future applications.

Table 3. Hybridization studies with 9-mer, 10-mer and 11-mer oxy-LNA and α-L-LNA towards complementary DNA and RNA. A = nucleotide monomer with an adenin-9-yl base, C = nucleotide monomer with a cytosin-1-yl base, G = nucleotide monomer with a guanin-9-yl base, T = nucleotide monomer with a thymin-1-yl base. ^{Me}C = nucleotide monomer with a 5-methylcytosin-1-yl base. Oligo-2′-deoxyribonucleotide sequences are depicted as d(sequence), oligoribonucleotide sequences as r(sequence) and 2′-O-Me-oligoribonucleotide residues are underlined. LNA monomers are shown in boldface with superscript 'L'. Superscript αL indicates an α-L-LNA residue.

Entry	Sequence	DNA target T_m (°C)	RNA target T_m (°C)
1 Reference DNA	5′-d(T)$_{14}$	32	28
2 Oxy-LNA	5′-d(**T^{L}**)$_9$T	80	71
3 α-L-LNA	5′-d(**$^{\alpha L}$T^{L}**)$_9$T	63	66
4 α-L-LNA/oxy-LNA chimera	5′-d(T)$_3$(**T^{L}**)$_4$(**$^{\alpha L}$T^{L}**)$_4$(T)$_5$	36	46
5 Reference DNA	5′-d(GTGATATGC)	28	28
6 α-L-LNA/DNA chimera	5′-d(G**$^{\alpha L}$T^{L}**GA**$^{\alpha L}$T^{L}**A**$^{\alpha L}$T^{L}**GC)	37	45
7 α-L-LNA/2′-OMe-RNA chimera	5′-(G**$^{\alpha L}$T^{L}**GA**$^{\alpha L}$T^{L}**A**$^{\alpha L}$T^{L}**GC)	38	52
8 Reference DNA	5′-d(CACACTCAATA)-3′	36	30
9 Oxy-LNA/DNA chimera	5′-d(C**A^{L}**C**A^{L}**C**T^{L}**C**A^{L}**A**T^{L}**A)-3′	54	63
10 α-L-LNA/DNA chimera	5′-d(C**$^{\alpha L}$A^{L}**C**$^{\alpha L}$A^{L}**C**$^{\alpha L}$T^{L}**C**$^{\alpha L}$A^{L}**A**$^{\alpha L}$T^{L}**A)-3′	39	49
11 Fully modified oxy-LNA	5′-(**^{Me}CA^{Me}CA^{Me}CT^{Me}CAATA**)^{L}-3′	69	77
12 Fully modified α-L-LNA	5′-$^{\alpha L}$(**^{Me}CA^{Me}CA^{Me}CT^{Me}CAATA**)^{L}-3′	65	75

Consequently, out of the many described LNA diastereoisomers I will focus on the α-L-LNA in this section [14–21, 27, 30].

Just like the oxy-LNA the α-L-LNA can be incorporated in complex designed oligo chimerae and act co-operatively with the non-LNA residues to induce increased affinity (e.g. table 3, entry 7). However, the affinity increase is generally not as high as for oxy-LNA. α-L-LNA can also be incorporated in oligos containing both DNA and oxy-LNA residues (table 3, entry 4).

The α-L-LNA and the oxy-LNA exhibit in many cases the same hybridization pattern towards DNA and RNA. This holds for sequences containing only a few LNA residues, 'spiked' sequences (table 3, entries 6, 7 and 10), and for fully modified sequences (table 3, entry 11 and 12). For homo-thymine sequences oxy-LNA and α-L-LNA exhibit about the same affinity for RNA but oxy-LNA is significantly more stable towards DNA (table 3, entries 2 and 3). For some spiked mixed sequences α-L-LNA induces only a modest affinity increase for DNA (table 3, entries 6, 7 and 10) whereas the affinity increase induced toward RNA is significant. This hybridization property is an interesting feature of α-L-LNA because RNA, and not DNA, is an important target in many biological applications. One application in which RNA binding preference is wanted is in the development of efficient and specific antisense drugs [31, 32]. It should be mentioned that the hybridization selectivity of α-L-LNA is the same as found for oxy-LNA [19].

Both oxy-LNA and α-L-LNA have a locked 3′-*endo* conformation (N-type)[1] (figure 6). Molecular modelling shows that that atoms of the 5′-OH and 3′-OH groups on the furanose and the N1 of the nucleobase are positioned at approximately the same co-ordinates in space for both the oxy-LNA and the α-L-LNA [20]. The positions of 5′-OH and 3′-OH are determining for the inter-nucleoside contact and the position of N1 determines the position

[1] The C3′-*endo* furanose conformation is in the case of α-L-LNA equivalent to an N-type conformation because of the L-configuration.

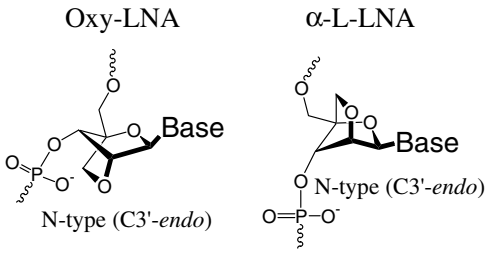

Figure 6. Oxy- and α-L-LNA.

of the nucleobase relative to the inter-nucleoside contacts. Therefore, despite the apparent differences in the chemical structure of the two LNA molecules, the position in space of the central atomic coordinates is almost the same. This is at least in part an explanation for the similar hybridization properties between the two LNA relatives. It is also interesting to note that the inter-nucleoside space in an LNA/DNA hybrid can accommodate the bicyclic structure no matter whether it points into the major groove (α-L-LNA) or it points into the minor groove (oxy-LNA). There is no doubt that the determination of these spatial co-ordinates is essential for the design of DNA analogues having improved binding properties to nucleic acids.

3. Hybridization kinetics

It is generally believed that duplex formation of oligonucleotides is a process involving two main events, initial formation of a nucleation complex followed by annealing of the duplex,

$$\mathrm{DNA(A) + LNA(B)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{IC} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{D}$$

where IC is the nucleation complex and D the duplex. The temperature-dependent overall dissociation constant, K_D, is given by

$$K_\mathrm{D} = (k_{-1} \times k_{-2})/(k_1 \times k_2) = [\mathrm{DNA\text{-}A}_{eq}][\mathrm{DNA\text{-}B}_{eq}]/[\mathrm{D}].$$

The hybridization kinetics of oxy-LNA oligonucleotides containing oxy-LNA thymines has been studied [33]. Christensen *et al* conducted a series of stopped-flow experiments using the following oligonucleotides:

DNA-1 : 5′-TGCTCCTG-3′
LNA-1 : 5′-T$^\mathrm{L}$GCTCCTG-3′
LNA-2 : 5′-T$^\mathrm{L}$GCT$^\mathrm{L}$CCTG-3′
LNA-3 : 5′-T$^\mathrm{L}$GCT$^\mathrm{L}$CCT$^\mathrm{L}$G-3′.

The observed rate constants were found to be of the order of 2×10^7 ($\mathrm{M^{-1}s^{-1}}$) (table 4) and the overall K_D values were calculated from the T_m curves at various temperatures.

ΔH was determined by plotting $\log K_\mathrm{D}$ as a function of the reciprocal temperature and ΔG was determined by the Gibbs–Helmholtz equation. The reported thermodynamic values were significantly higher for LNA oligonucleotides compared to DNA and this is in accordance with the very stable DNA hybridization formed by LNA. It was clearly demonstrated that the *on*-rates were similar for DNA–DNA and DNA–LNA hybridization. Therefore, the very different K_D found between DNA and LNA results in a marked difference in the *off*-rate. By taking the fast *on*-rates into account it was estimated the hybridization is nearly diffusion controlled, meaning that every correct base-to-base encounter leads to hybridization. The rate-determining step is the association reaction followed by fast annealing of the duplex.

Table 4. Hybridization kinetics and thermodynamics of oxy-LNA/DNA chimeric oligonucleotides against DNA complements. The thermodynamic values shown in the table are for duplex dissociation.

Reactants	T (°C)	k_{obs} (M^{-1} s^{-1})	K_D (nM)	k_{off} (s^{-1})	T_m (°C)	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ K^{-1} mol^{-1})
DNA–DNA	25	2.0×10^7	10	0.2	35	45	251	206
DNA–LNA(1)	25	1.9×10^7	20	0.4	34	44	254	210
DNA–LNA(2)	25	2.1×10^7	2	0.04	40	50	268	218
DNA–LNA(3)	25	2.4×10^7	0.3	0.006	45	55	302	247

4. Conclusion

In this paper it has been shown that LNA is a family of compounds. Most members of this family share the attractive high affinity binding properties first described for the parent oxy-LNA. It is equally important to note that the LNA members discriminate between perfect base pair matches and base pair mis-matches at least as well as DNA.

The bicyclic structure of LNA renders the oligonucleotides structurally different from nucleic acids [20, 34–36]. It has been shown that oxy-LNA oligonucleotides are stable in biological fluids [37]. The charged phosphate backbone of LNA oligonucleotides means that LNA oligonucleotides can be transfected and taken up by cells just like DNA. These properties are very important for many biological applications [38–47].

It has been a long sought goal to develop drugs with the capacity to control malignant gene expression base specifically. The applications of such drugs in e.g. cancer and infectious diseases are self-evident. Native oligonucleotides cannot be employed as such mainly due to their instability in cellular media and to low affinity for the target genes. The wish to develop nucleic acid probes with improved properties in this regard has been the main driver behind the massive synthesis effort in the area of nucleic acid analogue research. To this end LNA represents a very promising class of molecules. The basic properties described above combined with the fact that LNA (oxy) has been found to be non-toxic in animal models warrants a lot of promise of the molecule in novel medicines [31, 37, 44].

The basic hybridization properties shared by the LNA family members combined with the differences that each member has is a unique property position for LNA to fine tune drugs with very specific target potential.

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