This chapter presents a brief review about the advancements in nucleic acid therapeutics with respect to different aspects of antisense oligonucleotides for selective inhibition of gene expression. The oligonucleotide therapeutic agents such as antisense DNA, ribozymes, siRNA, and miRNA, act on target mRNA and arrest the protein synthesis. The over production or abnormal production of proteins is implicated or associated with many diseases. Antisense technology prevents the production of disease causing protein which results in a therapeutic benefit to patients. This chapter also describes the relevant literature pertaining to the useful chemical modifications of DNA/RNA with a particular emphasis on genetic 3′-5′ and nongenetic 2′-5′ linked nucleic acids and their structural features.
1.1 Introduction

Antisense agents are valuable tools for inhibiting the expression of target genes in a sequence-specific manner, and have potential in therapeutics. The antisense oligonucleotides (AS-ONs) exhibit either corrective or disruptive activity through strong and specific interaction with target mRNA sequences. The disruptive antisense ONs down-regulate the synthesis of disease-causing functional proteins via various mechanisms like steric blocking of ribosomal machinery, RNase H susceptibility, ribozyme activation and/or RNA interference induced by short double stranded RNA molecules like siRNA/miRNA. In the case of corrective antisense ONs, restoration of the viable protein synthesis is effected by acting at pre-mRNA level via splice corrections. The research activity in this area is directed towards improving practical difficulties encountered by AS-ONs such as cellular delivery, intracellular stability, ability to hybridize with the target sequences, susceptibility to RNase H cleavage, ease of synthesis and safety.

To overcome these challenges several chemical modifications of DNA/RNA have been developed. The ONs having ‘nongenetic’ 2′-5′ phosphodiester linkages (ngDNA) were also considered as candidates for antisense applications due to their RNA selective hybridization properties and enzymatic stability.

In this chapter we describe the literature pertaining to the different antisense strategies, AS-ONs and chemical modifications, structural features, binding preferences and recent developments in the area of DNA and ngDNA.

1.2 Nucleic acids

1.2.1 Genetic and non genetic nucleic acids: Structural overview

DNA and RNA as genetic material is polymer of nucleotide units, and each nucleotide is a micromolecular component comprising of a heterocyclic nitrogenous base, a β-D-pentafuranose sugar moiety and a phosphodiester linkage (Figure 1). The heterocyclic bases are purines adenine (A) and guanine (G) and pyrimidines cytosine (C), thymine (T) and uracil (U). The difference in DNA and RNA is whether the pentose is 2′-deoxyribose (DNA) or ribose (RNA) and the thymine (T) is replaced by uracil (U) in RNA (Figure 1). The heterocyclic base is covalently linked (at N-1 of pyrimidines and N-9 of purines) via a
**Chapter 1**

Introduction

*N-β*-glycosyl bond to the 1′ carbon of the pentose. The phosphate forms a diester linkage between the 3′ and 5′-hydroxy groups of the adjacent sugars forming the biopolymer. In the natural DNA and RNA, the D-sugar enantiomer and β-glycosyl linkages are present over the L- and α-counterparts.

![Diagram of DNA/RNA and ngDNA/ngRNA structures](image)

**Figure 1.** Genetic and non genetic nucleic acids

Natural nucleic acids with 3′-5′ phosphodiester linkages (Figure 1) function in encoding, transmitting and expressing genetic information, therefore are referred to as genetic nucleic acids. In most cases DNA carries the hereditary information from generation to generation and RNA functions as messenger to convert the genetic information into amino acid sequences which form functional proteins.

The nucleic acids with 2′-5′ phosphodiester linkages are also found in nature but they are not used to encode genetic information and hence are called as nongenetic nucleic acids (ngDNA, ngRNA) (Figure 1).

In 1953, J. D. Watson and F. H. Crick discovered the double stranded structure of DNA (excluding viruses) (Figure 2a). The nitrogenous base pairs in DNA (A–T and C–G) are formed via hydrogen bonds within the antiparallel double-stranded (ds) structure for DNA. The N-H groups of the bases are the hydrogen donors, while the sp²-hybridized electron pairs on the oxygen’s of the base C=O groups and the ring nitrogen’s are hydrogen...
bond acceptors, which are engaged in the formation of specific (Watson–Crick) A-T and G-C base pairs (Figure 2b).\(^2\) A–T pairs form two hydrogen bonds while C–G pairs form three. RNA usually exists in the single-stranded (ss) form but may fold into secondary and tertiary structures through the formation of specific base pairings (A-U and C-G).

![Figure 2. (a) Structure of Double-stranded DNA (B-DNA) (b) Watson and Crick hydrogen-bonding scheme for A: T and G: C base pair](image)

Other significant base pairing are the Hoogsteen\(^3\) and Wobble\(^3\) base pairs. In Hoogsteen base pairing, the purine rotates 180° with respect to the helix axis and adopts syn conformation. Hoogsteen base pairs utilize the C6-N7 face of the purine for hydrogen bonding with Watson-Crick (N3-C4) face of the pyrimidine (Figure 3, a and b). This base pairing permits the formation of triple helix DNA also called triplex or H-DNA. In Wobble base pairing, a single purine is able to recognize a non complementary pyrimidine (e.g. IU and GU, where I= Inosine, U=Uracil) (Figure 3, c and d) and these have importance in the interaction of messenger RNA (mRNA) with transfer RNA (tRNA) on the ribosome during protein synthesis (codon-anticodon interactions). In ONs, the base pairs stack over each other (\(\pi-\pi\) interaction) further stabilizing the secondary structure.
Figure 3. Hoogsteen (a,b) and Wobble (c,d) hydrogen-bonding

1.2.2 DNA Conformations

The conformations of nucleic acids are dynamic and are stabilized by several forces. Besides base pairing, the conformational features are constrained by pseudo-torsional angles for rotation around each bond. The details of the conformational structure of nucleotides are accurately defined by the torsional angles $\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$ and $\zeta$ in the phosphate backbone, $\nu_0$ to $\nu_4$, in the furanose ring, and $\chi$ for the glycosidic bond (Figure 4).\(^4\)

Figure 4. Torsional angle notations for one nucleotide

The six sugar phosphate backbone torsion angles [$\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$ and $\zeta$] are often roughly described as being in a conformational hyperspace such as cis ($c$) = $0 \pm 30^\circ$, + gauche ($g^+$) = $60 \pm 30^\circ$, + anticlinal ($a^+$) = $120 \pm 30^\circ$, trans ($t$) = $180 \pm 30^\circ$, - anticlinal ($a^-$) = $240 \pm 30^\circ$, - gauche ($g^-$) = $300 \pm 30^\circ$. 
The base is attached to sugar by a glycosidic bond. The torsion angle about this bond is defined as $\chi$ [O4'-C1'-N9-C4] for purines and [O4'-C1'-N1-C2] for pyrimidines. Torsion $\chi$ can adopt two main conformation called syn ($-90^\circ \leq \chi \leq 90^\circ$) and anti ($90^\circ \leq \chi \leq 180^\circ$). Anti orientation is more favorable compared to syn orientation because in the anti conformation there is no particular steric hindrance between sugar and nucleobase. However, the angle of $\chi$ can be tuned by the sugar pucker as well as the base type (purine or pyrimidine) and substituent on the base (adenosine is in anti conformation but 8-bromoadenosine is in syn conformation).

A planar conformation is energetically unfavorable for the pentafuranose sugar moiety of nucleotide because in this arrangement, all the substituents attached to carbon atoms are fully eclipsed. The system reduces its energy by puckering. The ring puckering arises from the effect of nonbonded interactions between substituents at the four ring carbon atoms—the energetically most stable conformation for the ring has all substituents as far apart as possible. This ‘puckering’ is described by identifying the major displacement of the carbons C2' and C3' from the median plane of C1'-O4'-C4'. Thus, if the endo-displacement of C-2' is greater than the exo-displacement of C3', the conformation is called C2'-endo and so on for other atoms of the ring. The endo-face of the furanose is on the same side as C5' and the base; the exo-face is on the opposite face to the base (Figure 5).

![Figure 5. N-type and S-type sugar puckering](image)

The concept of pseudorotation has been introduced to describe the conformation of ribose and deoxyribose rings in nucleotides with two variable parameters, the pseudorotation phase angle $P$ and the puckering amplitude $v_{\text{max}}$. The both parameters $P$ ($P = (v_4+v_1)-(v_3+v_0)/2v_2 (\sin36+\sin72)$) and $v_{\text{max}}$ ($v_{\text{max}} = v_2/\cos P$) is depends upon the furanose ring torsion angle $v_0$ to $v_4$. Under physiological conditions, the deoxyribofuranosyl sugars in DNA adopt preferentially 3'-exo, 2'-endo twist form (often referred to as South or S-type conformation), whereas ribofuranosyl sugars in RNA are in 3'-endo, 2'-exo twist form.
(often referred to as North or N-type conformation). Interestingly the conformation predominantly adopted by a nucleoside/nucleotide is not always present in a oligonucleotide double helix. In solution, N-type and S-type conformations are in rapid equilibrium and are separated by a low energy barrier (Figure 5). The pseudorotation cycle of sugar part of nucleoside is shown in Figure 6.7

![Figure 6. Pseudorotation cycle of the furanose ring in nucleosides. E = envelope; T = twist. The North type \( (1^\circ \leq P \leq 34^\circ) \) and South type \( (137^\circ \leq P \leq 194^\circ) \) pseudorotamers commonly populated in \( \beta\)-D-oligonucleotides are shaded.]

1.2.3 Stereoelectronic effects in sugar conformation of nucleosides

The conformation of the pentofuranose sugars are not static in solution and are involved in two-state North→South equilibrium (Figure 5). The dynamic nature of this equilibrium was evidenced by Chattopadhyaya’s group8,9 by temperature-dependent NMR studies, which showed that conformation adopted by a sugar depends upon a number of factors, such as the anomeric effect, gauche effect and steric interactions.

In terms of steric effect alone, for \( \beta\)-D-nucleosides, S-type pseudorotamers are energetically favored in comparison with N-type counterparts, since the pseudoequatorial oriented nucleobase in the former exerts less steric repulsions with the other substituents on the pentofuranose moiety than when it is pseudoaxial in the latter (Figure 7). But the energetics of N→S conformational equilibrium is known to be determined by the relative strengths of anomeric and gauche effects.
The O4'-C1'-N1/9 anomeric effect or Edward-Lemieux effect received its name from the term used to designate the C-1 carbon of a pyranose/furanose, the anomeric carbon. It is a stereoelectronic effect that describes the tendency of heteroatomic substituents adjacent to a heteroatom within a cyclohexane/cyclopentane ring to prefer the axial orientation instead of the less hindered equatorial orientation that would be expected from steric considerations. This is due to the stabilizing interaction (hyperconjugation) between the unshared electron pair on the one heteroatom (the endocyclic one in a sugar ring) and the $\sigma^*$ orbital for the axial (exocyclic) C–X bond (Figure 8).

The heterocyclic bases are involved in the anomeric effect by orbital mixing of one of the nonbonded lone pairs to the $\sigma^*$ orbital of the glycosyl bond ($n \ (O4') \rightarrow \sigma^* \ C-N$) to drive the two-state N↔S pseudorotational equilibrium of the constituent $\beta$-D-pentofuranosyl moieties. The anomeric effect in nucleosides is optimal in O4'-exo (W) conformation with the pseudoaxial aglycone, but this optimal conformation is unacceptable for steric reasons. The N-type conformation is energetically favored over S-type in terms of the anomeric effect alone, and its strength is closely related to the electronic nature of the aglycone. The protonation of the nucleobase facilitates the $n \ (O4') \rightarrow \sigma^* \ C-N$ interactions, thereby resulting in the increased population of N-type conformers.
The term "gauche" refers to conformational isomers (conformers) where two vicinal groups are separated by a 60° torsion angle and hinder bond rotation. Gauche rotamer are more stable than the anti rotamer due to hyperconjugation.

For example this effect is present in 1,2-difluoroethane (H₂FCCFH₂), where in the hyperconjugation model, the donation of electron density from the C–H σ bonding orbital to the C–F σ* antibonding orbital is considered the source of stabilization in the gauche isomer. Due to the greater electronegativity of fluorine, the C–H σ orbital is a better electron donor than the C–F σ orbital, while the C–F σ* orbital is a better electron acceptor than the C–H σ* orbital. Only the gauche conformation allows good overlap between the better donor and the better acceptor (Figure 9).

The 3'-OH or 3'-OPO₃H groups in 2'-deoxynucleosides, and 2'-deoxynucleotides respectively in DNA drive the N↔S equilibrium toward S-type conformation through the tendency to adopt a gauche orientation of the [O₄'-C₄'-C₃'-O₃'] torsion angle. The 2'-OH in the ribonucleosides and ribonucleotides in RNA is involved in three gauche interactions which compete for the drive of N↔S equilibrium: (i) [O₄'-C₁'-C₂'-O₂'] drives toward N, (ii) [N₁/9-C₁'-C₂'-O₂'] drives toward S, and (iii) [O₃'-C₃'-C₂'-O₂'] adopts gauche orientation in both N- and S-type sugar conformations (Figure 10).

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**Figure 9.** 1, 2-difluoroethane conformers

**Figure 10.** The 3'-OH or 3'-OPO₃H- groups in DNA drive the N↔S equilibrium toward S-type conformation through gauche effect
1.2.4 Helical structure of nucleic acids

Under physiological conditions, DNA:DNA duplex exists in the B-form while DNA:RNA and RNA:RNA duplexes predominantly adopt the A-form. Both the A- and B-form exist as an anti-parallel right handed helix (Figure 11).2,10

![Figure 11](image-url)

**Figure 11.** Double helical structure of B-forms DNA:DNA and A-form DNA:RNA/RNA:RNA. Sugar conformations in two-state North ↔ South equilibrium

An extremely important difference lies in the sugar conformation of the two forms of DNA, in the A-form the sugars adopt the C3'-endo conformation whereas in the B-form the conformation is C2'–endo (Figure 6). In A-DNA, the 2'–OH group in RNA single strand allows sugar puckers to be predominantly N-type because of anomeric and gauche effects as this structurally rigid RNA strand brings about the preferential N-type sugar conformations for the sugar in DNA strand of DNA:RNA duplex. Some other differences include the number of base pairs per helical turn (10 versus 11 in B and A forms, respectively), the bases being perpendicular to the helical axis in B form and slightly tilted in the A form. Also distinctly visible in the different forms are two grooves, which can act as possible hydrogen bond acceptors and donors. The major groove of A-DNA is narrow and deep, while in B-DNA, it is wide and deeper. The minor groove of A-DNA is broad and shallow, whereas in B-DNA, it is deep and narrow.
1.3 Antisense technology

Genetic information flows from DNA to RNA in a process called transcription, and is then used to synthesize proteins by a process called translation. RNA synthesis is initiated by transcription which first synthesizes precursor-mRNA. The pre-mRNA is composed of exons and introns (intervening sequences). However, introns are not translated into the amino-acid chain of a protein. In order to produce a functional protein these introns are removed from the pre-mRNA by splicing and the coding sequences (exons, carrying the genetic information) are fused together and form mature mRNA. The mRNA is then transported to the cytoplasm where it undergoes 'translation' with the help of other RNA like tRNA (transfer RNA) and rRNA (ribosomal RNA) to produce proteins.

Conventional drugs bind to proteins related to the disease and thereby modulate their function to curb the diseased state. An alternative treatment is known as ‘antisense therapy’. The antisense therapy involves inhibition of mRNA translation to proteins by using ssONs (DNA or RNA) having sequence complementary to their target mRNA. The term 'antisense' was coined to describe ONs that bind to the 'sense' mRNA in an anti-parallel, sequence specific manner via Watson-Crick hydrogen bonding. Antisense drugs could be selective and they have the potential to be more effective and less toxic than conventional drugs. The potential of ONs to act as antisense agents that inhibit viral replication in cell culture was discovered by Zamecnik and Stephenson in 1978. Antisense technology has rapidly developed and been widely applied for investigating gene function and regulation, modulation of gene expression, and validation of new drug targets. The first antisense drug, Vitravene, has been approved for the treatment of patients with cytomegalovirus- induced retinitis. Several AS-ONs have entered phase I–III clinical trials as anticancer agents administered alone or in combination with conventional chemotherapy.

The AS-ONs exhibit either corrective or disruptive activity through strong and specific interaction with target mRNA sequences. The disruptive AS-ONs down-regulates the synthesis of disease-causing functional proteins by 'translational arrest' via steric blocking of ribosomal machinery, degradation of the mRNA by activation of RNA cleaving
enzymes such as RNase H or RNase L, ribozyme activation and/or RNA interference induced by small interfering RNA molecules like siRNA/miRNA (Figure 12)\textsuperscript{14}.

![Figure 12. Various antisense mechanisms for inhibition/controlling of gene expression](image)

In the former mode of action, AS-ONs strongly associate to the untranslated region of the mRNA and prevent translation. It has been shown that AS-ONs acting via 'translational arrest' are readily unwound by the ribosome and do not block mRNA translation when targeted within the coding region. Nevertheless, a high affinity AS-ONs can overcome the unwinding activity of the initiation complex when targeted upstream to the coding region.

An alternative mode of action is by activation of RNA cleaving enzymes, such as RNase H and RNase L. RNase H is an ubiquitous enzyme that cleaves the RNA strand of a RNA:DNA hybrid duplex if the AS-ONs is structurally similar to DNA or the AS-ON:RNA duplex adopts a structure that resembles the DNA:RNA duplex. A second class of enzyme activating AS-ONs are the 2'-5'-linked adenylate-antisense chimera, that lead to mRNA degradation, by activation of a cellular enzyme called RNase L. The 2'-5'-A-antisense chimeras consists of a 2'-5' linked tetra-adenylate (2'-5'A)\textsubscript{4} linked to an AS-ONs via a flexible alkyl linker. The antisense oligonucleotide sequence binds to the mRNA
selectively and the 2′-5′ adenylate portion activates RNase L leading to the localized RNA degradation of the RNA:AS-ONs duplex.

RNA interference (RNAi) also called post transcriptional gene silencing (PTGS), is a biological process in which small interfering double-stranded RNA (siRNA) molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. In the siRNA pathway, the endonuclease enzyme DICER, binds to siRNA and helps it to load into RISC (RNA induced silencing complex) by forming RISC loading complex (RLC). During the activation of the RISC, the passenger strand (or sense) strand is cleaved, while guide strand (or antisense) strand, remains in the RISC.\textsuperscript{15} The actual effector of the RNAi is the ribonucleoprotein complex RISC, which is guided by the siRNA to the complementary target RNA. As a result, the target RNA is cleaved at a specific site by catalytic protein Argonaut 2 (Ago2).\textsuperscript{16} The cleaved mRNA is rapidly degraded by RNases and the coded protein can no longer be synthesized.

In the case of corrective antisense strategy, restoration of the viable protein synthesis is effected by acting at pre-mRNA levels for splice corrections (Figure 13).\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Simple illustration of exons and introns in pre-mRNA. The mature mRNA is formed by splicing. (UTR: Untranslated region)}
\end{figure}

1.4 Antisense oligonucleotides: Improvement through novel chemical modifications

The, AS-ONs requires strong and specific interactions with target mRNA sequences for their corrective as well as disruptive antisense activity. The platform where this interaction occurs may be either cytoplasm or nucleus and AS-ONs may have to compete with isosequential DNA for complex formation. Thus, ONs that would bind sequence specifically to RNA backbone assume importance.

Unmodified AS-ONs are rapidly attacked by nucleases in a biological system and being negatively charged their penetration through the cell membrane is not very effective. Several chemical modifications have been developed to enhance the nuclease resistance,
increase affinity and potency, of these AS-ONs. The general sites of modification are deoxyribo- or ribo- sugars, phosphate backbones, heterocyclic bases or complete replacement of the sugar phosphate backbone (Figure 14a). A vast number of chemically modified nucleotides have been used in antisense experiments most of which have been described in the recent review articles.18

The backbone modified ONs called 'first generation' antisense ONs, in which the phosphodiester linkage has been replaced with an alternate moiety. Examples of these include phosphorothioates,19 boranophosphates,20 phosphorodithioates, phosphoramidates21 and methylphosphonates,22 etc. Among these only first three are able to activate RNase H based degradation of RNA. These modifications enhance the stability towards nucleolytic hydrolysis, but decreases the stability of duplexes formed with complementary DNA or RNA than the natural phosphodiester linkage. The phosphorothioates were the first modified ONs resulting in a FDA approved drug named Vitravene.

The modification in heterocyclic bases is found to be very less in order to prevent any possible negative impact upon their hydrogen bonding and base stacking ability, both of which are important for recognition and duplex formation. It has been observed that C-5 substitution (methyl) in pyrimidines generally results in an improvement of the desired properties.6a,23

Figure 14: a) Modifications sites on an oligonucleotide dimer unit b) Different chemical modifications in genetic nucleic acids
The second generation of antisense ONs involve the modifications of sugar. Sugar modifications that favour a $C'_{3'}$-endo or north (N) conformation, typically found in RNA, tend to form the most stable duplexes. In DNA the gauche effect between $O_{3'}$ and $O_{4'}$ results in a $C_{2'}$-endo or south (S) sugar conformation. By modifying the sugar with electronegative substituents at the $C_{2'}$ and $C_{3'}$ position the overall conformation of the sugar can be locked or frozen to yield ONs that bind with high affinity. Examples of sugar modified ONs are the $2'$-$O$-methoxy ($2'$-$O$-Me),$^{24}$ arabinofuranosyl (ANA),$^{25}$ and $2'$-fluoroarabinofuranosyl ($2'$-F-ANA),$^{26}$ $2'$-fluororibofuranosyl ($2'$-F-RNA) etc.$^{27}$

Locked nucleic acids (LNA/BNA)$^{28,29}$ are a class of high-affinity RNA analogs in which the ribose ring is “locked” in $3'$-endo (N-type) conformation by $2'$-$O$,-$4'$-$C$-methylene bridge. In addition, these sugar modifications at the $2'$-position of RNA exhibit increased nuclease resistance, chemical stability against depurination and alter the lipophilicity, all of which contribute to improved pharmacokinetic properties. Some other examples of sugar modifications are hexitol nucleic acid (HNA),$^{30}$ cyclohexane nucleic acid (CNA), cyclohexenyl nucleic acid (CeNA),$^{31}$ and their several analogues. The third generation modification examples are where the sugar phosphate moiety has been completely replaced, like in peptide nucleic acids (PNA)$^{32}$ morpholino NA$^{33}$ and their analogues (Figure 14b).$^{34}$

We focus our attention on the ONs having ‘nongenetic’ $2'$-$5'$ phosphodiester linkages (ngDNA/ngRNA) and their modification due to their RNA selective hybridization properties and enzymatic stability.

1.5 $2'$-$5'$ Linked oligonucleotides: Nongenetic nucleic acids (ngRNA/ngDNA)

The $2'$-$5'$ linked constitutional isomer supports Watson–Crick base pair association but with distinct properties and are not used for biological information storage and transfer thus called nongenetic nucleic acids.$^{35}$

Almost all naturally occurring nucleic acids have $3'$-$5'$ phosphodiester linkages but studies suggest $2'$-$5'$ linkages were easier to form in the prebiotic world.$^{36}$ They are formed during intron splicing and also in interferon treated cells.$^{37}$ During nonenzymatic ONs synthesis, $2'$-$5'$ linkages are formed more easily than $3'$-$5'$ linkage.$^{38}$ It has been suggested
that 3'-5' linkages were selected during evolution because they hydrolyze slower than 2'-5' linkages, and/or the duplex structures formed by 2'-5' oligomers are less stable than those formed by 3'-5' oligomers. The 2'-5' linked ONs (ngDNA/ngRNA) offer high resistance to nuclease digestion and also exhibit remarkable selectivity for complexation with complementary single stranded RNA but not to DNA. This raises the possibility of the utility of 2'-5'-linked ONs in potential antisense and diagnostic applications.

2'-5'-linked oligoadenylates (2'-5'-Aₙ) were detected in a variety of cells and tissues, including L1210 cells and human lymphocytes. These oligoadenylates vary in length from 2 to 15 residues. The di-, tri- and tetra-adenylates are the most abundant products formed in the reaction and the amounts of larger oligoadenylate units diminish with increasing chain length. 2'-5'-Aₙ are responsible for antiviral mechanism of interferon (Interferons play a critical role in host immune responses against viral infections) and are also involved in cell growth regulation and differentiation. The biological action of the 2'-5'-Aₙ system is mediated by RNase L. The 2'-5'-Aₙ activate a ubiquitous endoribonuclease (RNase L), by binding to it and converting it from an inactive monomer to its catalytically active dimer which is then responsible for cleavage of RNA. RNase L is an enzyme found in many eukaryotic cells sometimes known as 2'-5'Aₙ dependent ribonuclease, is an interferon induced ribonuclease which, upon activation, destroys all RNA within the cell (both cellular and viral). For this reason, the 2'-5'Aₙ molecule is a potent inhibitor of translation, blocking cell-free protein synthesis at nanomolar concentrations.

Additionally, these 2'-5'Aₙ are also reported to inhibit the activities of HIV-1 reverse transcriptase and DNA topoisomerase-I in HIV-1-infected cells. After a conjugation with an antisense 3'-5' linked phosphodiester ONs, the 2'-5'Aₙ portion of the chimeric ONs can also direct antisense cleavage of target mRNA, presumably through activation of RNase L in cell culture.

1.5.1 Structural features of nongenetic nucleic acids (ngDNA/ngRNA)

In 1991, Damha et al. synthesized the 2'-5'-linked RNA (ngRNA Figure 1) that was found to exhibit self-pairing as well as pairing with RNA but its complexation with complementary DNA was not observed. The duplexes of ngRNA with complementary RNA (ngRNA:RNA) showed very similar CD patterns as the natural 3'-5' DNA:RNA
duplexes, from which it was deduced that the overall structures of these complexes would be comparable i.e., compact A-form duplex structure. NMR studies in solution for self-pairing ngRNA duplexes suggested a similar structure in which the ribose sugars assumed C2′-endo (S-type) puckering for the ngRNA strands. From the similarity found in the CD spectra of ngRNA:RNA and the self-pairing ngRNA duplexes, along with the NMR studies for the latter duplex, it was deduced that the ngRNA strand in the ngRNA:RNA duplex might also assume the structural features of a natural DNA strand and the sugar residues would adopt C2′-endo (S-type) conformations (Figure 15).

Very similar results were found independently by Breslow and Switzer for the 2′-5′-linked 3′-deoxyribonucleic acids (ngDNA) as well. CD spectroscopy, and modeling studies predicted that the sugar conformations in the ngDNA strand of ngDNA:RNA duplexes would have predominantly S-type sugar conformations. Contrary to this, the NMR studies on single-stranded ngRNA and 2′-5′-d(G4C4) implied an extended structure in which the sugar conformations were N-type. The C3′-endo or N-type sugar pucker has also been observed for single-stranded ngRNA in crystal structures.

![Image](image.png)

Figure 15: Sugar conformation of ngDNA nucleotide in single-stranded ngDNA/ngRNA and double stranded ngDNA/ngRNA:RNA A-form duplex

Molecular modeling studies reported by Yathindra et al. also have suggested that to arrive at the compact A-type overall structure of the ngRNA:RNA duplex, the S-type or C2′-endo geometry of repeating nucleotides is stereochemically favoured in the ngRNA strand of the duplex.
Thus, the structural requirements of the ngRNA/ngDNA:RNA duplexes (predominant S-type geometry of the 2'-5' strand in ngRNA/ngDNA:RNA duplex) do not match with the sugar conformations at the single stranded 2'-5' oligomer level. In contrast to DNA in the single-stranded 2'-5' oligomer, the stereoelectronic (gauche and anomeric) effects of the 2'-hydroxy group, would accentuate the preference for C3'-endo (N-type) sugar conformations. The N-type sugar pucker in 2'-5'oligomer leads to extended structure. While modelling the ngDNA:RNA duplex, it was therefore assumed that the RNA strand would impose its structure on the ngDNA strand, and the individual nucleosides in the isomeric DNA strand would be required to assume C2'-endo (S-type) conformation to give rise to a stable duplex. Thus, it was suggested that the 2'-5'-linked DNA would be under conformational and topological constraints when it forms duplex with complementary RNA.

Thus, it is postulated that geometry of 2'-5' and 3'-5' linkages are in an inverse relationship (Figure 16). A 2'-5' linkage with a C2'-endo sugar would produce a compact geometry favorable for RNA binding while the C3'-endo sugar would produce extended nucleotide geometry. In the case of 3'-5' linked oligomer the C2'-endo sugar would be in extended oligomer form, less suitable for RNA binding.

![Diagram](image)

**Figure 16**: 3'-5' DNA/RNA and 2'-5' ngDNA/ngRNA in compact and extended conformations showing inverse relationship.
1.5.2 Chemical modifications in nongenetic nucleic acids (ngRNA/ngDNA)

In ngDNA/ngRNA the linkage changes from C3' (as seen in DNA and RNA) to C2'. This leads to an increase in the number of bonds between O5' and phosphorus from six to seven. The experimental results suggest that an antiparallel duplex is formed by the ngDNA/ngRNA; however the overall stability is less than that of corresponding 3'-5' duplexes. The order of the thermal stability was found to be RNA:RNA > DNA:DNA ≈ RNA:DNA ≈ DNA:RNA > ngRNA:RNA ≈ ngDNA:RNA > ngRNA:ngRNA ≈ DNA:ngRNA. In addition, the ngDNA/ngRNA exhibit binding selectivity for 3'-5' RNA over 3'-5' DNA, and confer greater resistance to nucleolytic degradation as compared with the natural 3'-5' DNA/RNA. The diminished strength of RNA:ngRNA or RNA:ngDNA complexes as compared to DNA:RNA or RNA:RNA complexes and also the fact that ngRNA:RNA or ngDNA:RNA duplexes are not substrates of the enzyme RNase H could be the possible reason that these have not yet been exploited in therapeutic applications. In order to improve the binding strength, and RNA specificity, some efforts towards the modification in the 2'-5' nucleotides and their incorporation in mixed backbone oligonucleotide (MBO) containing ngDNA/RNA and 3'-5' DNA/RNA are found in literature. Very few chemical modifications in homogeneous ngDNA/ngRNA have been studied so far.

1.5.2a Backbone modifications

Backbone modifications in mixed 2'-5' and 3'-5' linked oligonucleotide

Mixed backbone oligonucleotide (MBOs) containing 2'-5'-ribonucleotides and 3'-5'-deoxyribonucleotides bind to both DNA and RNA target strands. The affinity of the MBOs for the DNA complementary strand decreases with increasing 2'-5'-ribonucleotide linkages. MBOs having ngRNA linkages at both end and 3'-5' DNA linkages at middle part of the sequence show lower thermal stability than control DNA and also show less nuclease resistance. The use of 3'-5' linkages with phosphorothioate backbone in the same sequence increased nuclease resistance but decreased thermal stability. 2'-5'/3'-5' DNA phosphate chimeras have been used to inhibit 5α reductase expression in cell culture. The single stranded DNA/ngRNA chimeras and ngDNA show less non-specific binding to plasma and cellular protein in comparison with 3'-5' PS-ONs hence produce fewer side effects in vivo.
The achiral uncharged phosphodiester replacement of 2′-5′ connection was explored by using 2′-5′ formacetal and thioformacetal linkages (Figure 17a). The TT dimer for 2′-5′ formacetal and thioformacetal were incorporated into a ONs of the sequence 5′-TC*TC*TC*TC*T^TT^TT-3′ where C*= 5-methyl-2′-deoxycytidine and T^T=2′-5′ modified thymidine dimers. The poor hybridization property of 2′-5′-linkages with DNA is confirmed in this study showing ΔTm of -8°C for only two 2′-5′ phosphodiester linkages which is only -2 °C for complementary RNA. The thioformacetal series shows a similar trend to the phosphodiester with the 2′-5′ connection resulting in particularly poor hybridization properties with a single strand DNA target (-7.5°C) and resulted in only a modest destabilization with RNA (-2.5°C). The 2′-5′ formacetal ONs results in comparable hybridization properties with DNA and RNA. The DNA binding properties show only modest (-2°C) destabilization relative to 3′-5′ phosphodiester and the RNA binding properties are nearly comparable to the 3′-5′ phosphodiester control for the 2′-5′ for formacetal (-1.0°C). Molecular modeling studies revealed reduced steric space for 2′-5′ phosphodiester linkage and the shorter non-bulky linkage between 2′ and 5′ positions was expected to be favored for both A and B conformations. The 2′-5′ thioformacetal derivative resulted in substantial destabilization of duplex formation with ssRNA and ssDNA. The 2′-5′ formacetal ONs resulted in only modest destabilization with ssDNA and ssRNA as compared to the control 3′-5′ phosphodiester ONs.

Figure17. Backbone modified ngDNA/RNA
Backbone modifications in homogeneous 2′-5′ linked oligonucleotide (ngDNA/ngRNA)

A phosphorothioate backbone modified homogeneous ngDNA sequence (Figure 17b)\textsuperscript{42} again like phosphodiester backbone binds specifically to RNA but do not hybridize to complementary DNA. PS-ngDNA results in little destabilization compare to its PO-ngDNA, but this destabilization is relatively less as compared to the destabilization into the PS-DNA than PO-DNA. Thus, it is clear that phosphorothioate substitution in ngDNA is less destabilizing in comparison with genetic DNA when hybridized to complementary RNA. Like PO-ngDNA, PS-ngDNA also does not activate RNase H. However, incorporation of seven 3′-5′ linked phosphorothioates in the center of PS-ngDNA generates a chimeric oligonucleotide that can support RNase H activity. The 2′-5′-linked PS-ONs show less non-specific binding with cellular proteins and thrombin in comparison with the 3′-5′-linked PS-ONs. A reduction in these non-sequence specific protein interactions with the 2′-5′-linked phosphorothioate motif may produce fewer of these side effects \textit{in vivo}.

In the A-type compact helices of ngRNA:RNA duplexes were shown to have smaller interstrand phosphate-phosphate distances and could have been the cause of destabilization. This point was considered by Peng and Damha,\textsuperscript{57} when they introduced an extra carbon atom in 2′-hydroxymethyl(hm)-2′-5′ RNA oligomers (Figure 17c, 2′-α-hm-5′RNA), in order to lengthen the backbone and reduce the interstrand P-P repulsion, still keeping compact geometry. This modification in homogeneous ngRNA sequence unfortunately destabilized the complex with RNA. Both ngRNA:RNA and ngRNA:ngRNA duplexes with a single modification with 1-(2-deoxy-2-α-C-hydroxymethyl-β-D-ribofuranosyl)thymine (2′-α-hm-dT) show a small loss in respective stability ($\Delta T_m$ ~ -0.8-0.9°C). When the number of modification units of 2′-α-hm-dT is increased to three the depression in $T_m$ is only -0.3 °C/modification, suggesting a stabilizing effect by the nearly continuous 2′-α-hm-dT residues. 2′-α-hm-dT monomer was also incorporated in 3′-5′-linked RNA sequence, but here the destabilization effect of 2′-α-hm-dT unit is greater ($\Delta T_m$ >-1 °C/modification) regardless of whether the target is complementary RNA or ngRNA. The 3′-5′ linked 2′-α-hm-dT monomer (Figure 17d) incorporated in homogeneous ngRNA but this also leads to reduced duplex stability with an equal destabilization with both complementary RNA and ngRNA.
1.5.2b Sugar modifications

Sugar modifications in mixed 2′-5′ and 3′-5′ linked oligonucleotide

The MBOs having five modifications in 3′-methoxy 2′-5′ -linked ONs (Figure 18-I)\(^{55}\) units at the both ends of the 20mer 3′-5′ DNA sequence show unique properties such as higher affinity for RNA, adoption of A-conformation and resistance to RNase H and nuclease S1 than unmodified sequence (five 2′-5′ -linked ONs at the both end of the 20 mer 3′-5′ DNA sequence). But the stability is still less compared to natural 3′-5′ linked ONs. While a slight decrease in \(T_m\) value for fully modified 3′-methoxy 2′-5′-linked ONs was observed and which also shows weaker resistance towards SVPD can be improved upon by introducing few phosphorothioate linkages at the 3′ end of the oligonucleotide.

3′-O-methoxyethyl-RNA (MOE-RNA) is another modification in 2′-5′ -linked ONs (Figure 18-II). The NMR studies revealed that the substitution of 3′-OH group in 2′-5′-linked ONs by 3′-methoxyethoxy (3′-MOE) group resulted in C3′-endo (N-type) conformation.\(^{53a}\)

![Figure 18: Sugar modified analogues of ngDNA/RNA](image)

Conformational constraint was introduced in 2′-5′-linked ONs in the form of a bicyclic 3′-O, 4′-C methylene ribonucleoside, (Figure 18-III) and 3′-amino-3′-4′-BNA (Figure 18-IV) in which predominant S-type conformation was predicted by NMR and PM3 calculation.\(^{58}\) One to six 2′-5′-linked modified monomer units were incorporated in
natural 3′-5′ ONs to check their binding affinity to the complementary DNA or RNA. The melting temperature (Tm) for the modified ONs shows marginal change (increase or decrease) in Tm towards complementary RNA as compared with the unmodified oligonucleotide, while moderate depression in Tm were observed towards complementary ssDNA. This indicates that the modified ONs with the conformationally restricted bicyclic 2′-5′ nucleoside analogues have significant selectivity for RNA over ssDNA. Incorporation of these analogues at the 3′-end of the ONs was shown to enhance the nuclease resistance.

The conformationally restricted 5, 5 bicyclic ribose 2′-5′ formacetal was identified as a candidate to pre-order a nucleoside to resemble the bound duplex A-type conformation. Trinucleoside bearing a two 2′-5′ formacetal linkages and a locked 3′, 6′-anhydro-glucose monomer (t′t≥c≥m) (Figure 18-V) was incorporated into an ONs sequence 5′-CMTTCMATTTT t′t≥c≥m -3′, where C M = 5-methyl-2′-deoxycytidine. The Tm for this sequence was compared with two control sequences, one is 5′-CMTTCMATTTTt′t′c′m-3′ which has t′t′c′m the non-conformationally restricted 2′-5′ formacetal-linked trimer and the remaining are 3′-5′ PO-linkages. The second sequence is 5′-CMTTCMATTTTTTCM-3′ having all 3′-5′ PO-linkages. The Tm of the ONs bearing two 2′-5′ formacetal linkages was 2.5 °C lower than that of corresponding with 3′-5′ phosphate and further modification by locked 5, 5 bicyclic ribose 2′-5′ formacetal moieties resulted in destabilization of the duplex with the target ssRNA compared to the 2′-5′ formacetal control. The rigid conformational restriction on the parent ribose ring and loss of flexibility to adopt a conformation suitable for duplex formation might have been the cause of this destabilization.

Thus from the current literature it appears that compact A-type conformation in 2′-5′ linked RNA could be C2′-endo and would favor the stabilization of the 2′-5′ isomeric DNA:RNA structures. Further changes/modifications need to be done that involve uniformity in the backbone, sequence variability etc. to clearly understand desired structural parameters for preferential RNA binding of 2′-5′ linked ONs.

Sugar modifications in homogeneous 2′-5′ linked oligonucleotide (ngDNA/ngRNA)

The inversion of configuration at 3′-OH in ngRNA, led to the synthesis of 2′-5′ xylose nucleic acids (ngXNA), (Figure 18-VI). The sugars of ngXNA adopt C3′-endo conformation due to two gauche effects along the O3′-C3′-C4′-O4′ and O2′-C2′-C1′-O4′, and
may therefore adopt an extended (DNA-like) conformation. The pre-organized extended conformation of ngXNA bound very weakly to the compact RNA target. The conformational incompatibility or spatial mismatching of two strands was thought to be the reason why extended XNA bound poorly to compact RNA.  

1.6 Spectroscopic techniques

Ultraviolet absorption (UV) and circular dichroism (CD) which looks into electronic properties of the bases and is highly useful and general tools for biophysical study of nucleic acids. MALDI TOF spectrometry is used as mass analysis technique.

1.6.1 Ultraviolet spectroscopy

The absorbance of polynucleotide depends on the sum of the absorbance of the nucleotide plus the effect of the interaction among the nucleotides. The interaction cause a single strand to absorb less than the sum of its nucleotides and a double strand absorbs less than its two component single strand. The effect is called hypochromicity which results from the coupling of the transition dipoles between neighbouring stacked bases and is larger in amplitude for A-U and A-T pairs.  

In converse term, hyperchromicity refers to the increase in absorption when a double stranded nucleic acid is dissociated into single strands. The UV absorption of a DNA duplex increases typically by 20-30 % when it is denatured. This transition from a stacked, hydrogen bonded double helix to an unstacked, strand-separated coil has a strong entropic component and is temperature dependent. The mid-point of this thermal transition is known as the melting temperature ($T_m$). Such a thermal dissociation of nucleic acid helices in solution to give single stranded DNA/RNA is a function of base composition, sequence, chain length as well as of temperature, salt concentration, and pH of the solvent (buffer). In particular, early observations of the relationship between $T_m$ and base composition for different DNAs showed that A-T pairs are less stable than G-C pairs, a fact which is now expressed in a linear correlation between $T_m$ and the gross composition of a DNA oligomer by the equation:

$$T_m = X + 0.41 \times (\% \text{C+G}) \degree \text{C}$$
The constant X is dependent on salt concentration and pH and has a value of 69.3°C for 0.3M sodium ions at pH 7.61.

A second consequence is that the steepness of the transition curve also depends on base sequence. Thus, melting curves for homooligomers have much sharper transitions than those for random-sequence oligomers. This is because A-T rich regions melt first to give unpaired regions which then extend gradually with rising temperature until; finally, even the pure G-C regions have melted. Short homooligomers melt at lower temperatures and with broader transitions than longer homooligomers. For example for poly (rA)n-poly (rU)n, the octamer melts at 9ºC, and the decamer at 20ºC, and long oligomers at 49ºC in the same sodium cacodylate buffer at pH 6.9. Consequently, in the design of synthetic self complementary duplexes for crystallization and X-ray structure determination, G-C pairs are often placed at the ends of hexamers and octamers to stop them ‘fraying’. The converse of melting is the renaturation of two separated complementary strands to form a correctly paired duplex.

**Figure 19:** An oligonucleotide duplex melting curve and derivative curve

**Duplex melting:** According to the ‘all or none model’62, the UV absorbance value at any given temperature is an average of the absorbance of duplex and single strands. A plot of absorbance against temperature gives a sigmoidal curve in case of duplexes and the midpoint of the sigmoidal curve (Figure 19) called as the ‘melting temperature’ ($T_m$) (equilibrium point) at which the duplex and the single strands exist in equal proportions.

**Triplex melting:** In the case of triplexes, the first dissociation leads to melting of triplex generating the duplex (WC duplex) and third strand (Hoogsteen strand), followed by the duplex dissociation to form two single strands. The DNA triplex melting shows characteristic double sigmoidal transition and UV melting temperature for each transition is...
obtained from the first derivative plots. The lower melting temperature ($T_{m1}$) corresponds to triplex to duplex transition while the second higher melting temperature ($T_{m2}$) corresponds to the transition of duplex to single strands.

1.6.2 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals. CD is particularly useful for studying chiral molecules and has very special significance in the characterization of biomolecules (including the secondary structure of proteins and the handedness of DNA). The commonly used units in current literature are the mean residue ellipticity (degree cm$^2$ dmol$^{-1}$) and the difference in molar extinction coefficients called as molar circular dichroism or $\Delta\varepsilon$ (liter mol$^{-1}$cm$^{-1}$). The molar ellipticity [$\theta$] is related to the difference in molar extinction coefficients by [$\theta$] = 3298 ($\Delta\varepsilon$). In the nucleic acid, the heterocyclic bases that are principal chromophores. As these bases are planar, they don’t have any intrinsic CD. CD arises from the asymmetry induced by linked sugar group. CD spectra from the dinucleotide exhibit hyperchromicity, being more intense by roughly an order of magnitude than those from monomers. It is when the bases are linked together in polynucleotide’s, giving rise to many degenerate interactions and they gain additional characteristic associated with the asymmetric feature of secondary structure such as in proteins and nucleic acids.

The simplest application of CD to DNA structure determination is for identification of polymorph present in the sample. The CD signature of the B-form DNA as read from longer to shorter wavelength is a positive band centered at 275nm, a negative band at 240nm, with cross over around 258nm. These two bands arise not from a simple degenerate exciton coupling, but as a result of superimposition of all coupling transitions from all the bases. A-DNA is characterized by a positive CD band centered at 260nm that is larger than the corresponding B-DNA band, a fairly intense negative band at 210nm and a very intense positive band at 190nm (Figure 20). The 250-230nm region is also usually fairly flat though not necessarily zero. Naturally occurring RNAs adopt the A-form if they are duplex.
Figure 20: CD spectra of DNA secondary structure

CD denaturation (melting) and CD-Job’s plots are equally important as they give $T_m$ and binding stoichiometry of DNA/RNA complexes respectively.

**Stoichiometry:** The binding *stoichiometry* of ONs to target DNA/RNA by UV-titration (mixing): This is derived from Job’s plot. The two components of the complex are mixed in different molar ratios, so that the total concentration of each mixture should be constant, i.e., as the concentration of one strand decreases, concentration of the second strand increases and the UV-absorbance of each mixture is recorded. The absorbance decreases in the beginning to a minimum and then again increases. The molar ratio of the two strands at which absorbance reached minimum indicates the stoichiometry of complexation.

### 1.6.3 Mass spectroscopy: MALDI-TOF

Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) is a soft ionization technique used in the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), where these large ions obtain in gas phase producing many fewer multiply charged ions. MALDI is a two step process. First, desorption is triggered by a UV laser (nitrogen lasers 337nm) beam. Matrix material heavily absorbs UV laser light, leading to the ablation of upper layer (~micron) of the matrix material. The hot plume produced during ablation contains many species: neutral and ionized matrix molecules,
protonated and deprotonated matrix molecules, matrix clusters and nanodroplets. Second, the analyte molecules are ionized (more accurately protonated or deprotonated) in the hot plume. The time-of-flight (TOF) analyzer uses an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If the particles all have the same charge, the kinetic energies will be identical, and their velocities will depend only on their masses. Lighter ions will reach the detector first. Their time-of-flight is proportional to their (MW)\(^{1/2}\).

The matrix is the crystallized, low molecular weight compound with several conjugated double bonds. The some basic properties of matrices is like they should not evaporate during standing in spectrometer, often acidic for acting as a proton source to encourage ionization of the analyte molecules, strong optical absorption in either the UV or IR range. Most commonly used matrices are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), \(\alpha\)-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix, CHCA) and 2, 5-dihydroxybenzoic acid (DHB). 2',4',6' trihydroxy acetophenone (THAP). A solution of one of these molecules is made, often in a mixture of highly purified water and an organic solvent (normally acetonitrile (ACN) or ethanol). trifluoroacetic acid (TFA) or ammonium citrate may also be added.

1.7 Present work

Oligoribonucleotides comprising of 2'-5'-linked internucleotide linkages are known to bind selectively to RNA over DNA. The ability to bind to RNA renders them suitable as probes for 'antisense technology'. Experiments to study the effect of sugar conformation on the binding properties of 2'-5'-linked ONs are not very well studied in the current literature. To get insight into the role of sugar conformations, 2'-5'-linked ONs modified at the C3'-position of the furanose ring were synthesized and their binding to complementary single stranded DNA and RNA was studied. Their nuclease resistance ability was also evaluated.

As we have seen that 2'-ribo and 2'-arabino-fluoro group has large but opposite impact on the sugar conformations. The increased thermal stability of the 3'-5' linked 2'F-RNA:RNA and 2'F-ANA:RNA duplexes is because of the favorable influence of the 2'-fluoro group on conformational restriction of the sugar pucker. The 3'-fluoro substitution of the furanose ring of nucleosides was therefore envisaged.
Chapter 1

The thesis describes synthesis and conformational analysis of S-type or N-type conformationally frozen uracil/thymine/adenine containing 2′-5′ nucleosides and S-type conformationally locked adenine 2′-5′ nucleoside. The incorporation of these modified units in RNA-selective, nuclease resistant 2′-5′-linked non-genetic deoxyribonucleic acid oligomers (ngDNA) and biophysical studies of these modified oligomers with target DNA/RNA sequences is discussed. Synthesis of compact arabino-uridine nucleoside, its incorporation in ngDNA and biophysical studies are presented briefly.

Chapter 2: The synthesis of S/N-type conformationally frozen, 3′-deoxy-3′-ribofluoro uridine (RUF) and 3′-deoxy-3′-xylofluoro (XUF) uridine nucleosides is described in this chapter. Synthesis of ngDNA containing these modified units and biophysical experiments to study the effects of these modifications on the stability of duplexes with target DNA/RNA are presented.

Chapter 3: This chapter is divided into 3 sections.

Section A: This section describes the synthesis of S/N-type-frozen 3′-deoxy-3′-ribo/xylofluoro thyminyl nucleosides (R^T^F and X^T^F) and S-type locked (LA^S) and S/N-type frozen 3′-deoxy-3′-fluoro ribo/xyloadenine nucleoside analogues (R^A^F, X^A^F) for their incorporation in ngDNA. Synthesis of ngDNA containing these modified units and biophysical experiments to study the effects of these modifications on the stability of duplexes with target DNA/RNA are presented.

Section B: This section describes the rationale for designing arabino-uridine (araU), its synthesis and incorporation into ngDNA oligomers. The effect of such modification on binding with target DNA and RNA is studied using temperature dependent UV-Tm experiments.

Section C: In this section we describe the nuclease resistance ability of the modified ngDNA oligomers in comparison with unmodified DNA and ngDNA by exposure to snake venom phosphodiesterase (SVPD).

Chapter 4: This chapter describes the conformational studies of RUF, R^A^F, XUF, X^A^F, LA^S, and araU monomers using NMR, X-ray crystal structure and Matlab pseudorotation GUI program.
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