

## Bridged Nucleic Acids (BNAs) as Molecular Tools

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### ABSTRACT

There has been a growing interest in developing chemically modified nucleotides for diagnostics or therapeutics. Among the list of the artificial nucleotides, bridged nucleic acids (BNAs) appear to be the most promising new generation BNAs to date in view of the usage for applications. Here we briefly introduce the modified nucleotides and the applications of the new generation BNAs. For the possible applications, BNAs can be used for antisense, antigene, aptamer development, and real-time clamp technology. Eventually, BNAs may play a pivotal role in a new type of disease diagnostics and therapeutics in the foreseeable future.

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**Key words:** BNA; Antisense; Aptamer; Real-time PCR clamp; Nucleic acid analogs

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### INTRODUCTION

With the completion of the human genome sequence, attention has been paid to the utilization of this sequencing information to genetic medicines, tailor-made medications and molecular targeted therapies. This has led to encourage scientists to develop artificial nucleic acid analogs. The artificial nucleic acid analogs potentially have many applications in molecular biology and genetic diagnostics and have emerged in the field of molecular medicine. The key importance of the artificial nucleic acid analogues is that when the modified oligonucleotides form DNA:DNA and DNA:RNA duplexes, the binding affinity is more stable than that of unmodified oligonucleotides<sup>[1-5]</sup>. Due to such an advantage, a variety of nucleic acid analogs have been synthesized to enhance high-affinity recognition of DNA and RNA targets and to increase duplex stability and increasing cellular uptake. However, although a large number of chemically modified oligonucleotides have been developed during the last few decades, most of these molecules have failed to give the desired response excluding a few molecules. Among the list of the initially successful nucleic acid analogs were peptide nucleic acids (PNAs), 2'-fluoro-3'-aminonucleic acids, 1', 5'-anhydrohexitol nucleic acids (HNAs), and locked nucleic acids (LNAs) (Figure 1). Peptide nucleic acids (PNAs) are synthetic polymers that contain a peptide backbone and nucleic acid bases as side chains<sup>[6,7]</sup>. These peptide based nucleic acid mimetic polymers can form strong specific hydrogen bonds with complementary sequences present in double-stranded DNA (dsDNA)<sup>[8]</sup>. 2'-fluoro-3'-aminonucleic acids are modified nucleotide analogs that contain fluorine at the 2' position<sup>[9]</sup>. HNAs are oligonucleotides built up from natural nucleobases and a phosphorylated 1,5-anhydrohexitol backbone, where 1',5'-anhydrohexitol oligonucleotides can be synthesized using phosphoramidite chemistry and standard protecting groups<sup>[10-14]</sup>. One dramatic improvement was made with the introduction of the bridged nucleic acid, 2', 4'-BNA, also called LNA<sup>[15]</sup>. The compound shows a better hybridization affinity for complementary strands, both for RNA and DNA strands, in comparison with unmodified nucleotides.

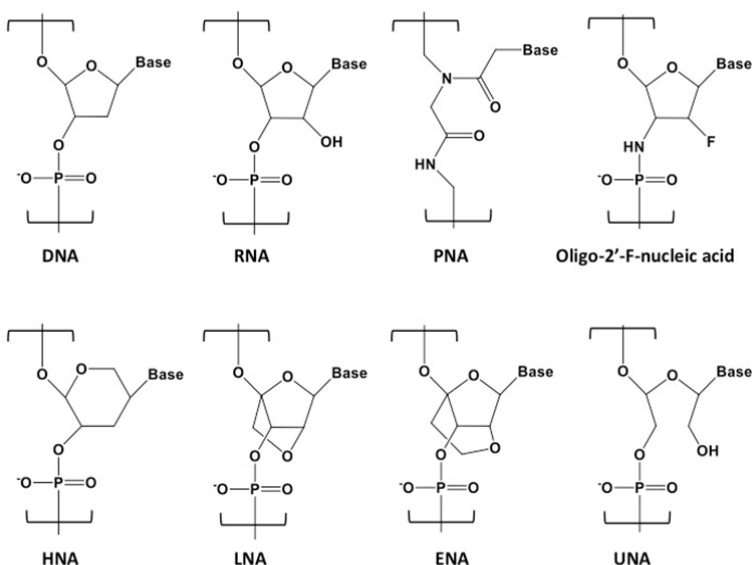
Furthermore LNA can be used to design sequence selective LNA-oligonucleotide hybrids that are soluble in aqueous solutions and exhibit improved biostability compared with natural nucleotides<sup>[16]</sup>. The LNA monomer has now been widely used in nucleic-acid-based technologies. However, there is a need for further development because the nuclease resistance of the LNA is significantly lower than that obtained by phosphorothioate oligonucleotide and the fact that oligonucleotides containing consecutive LNA units or a fully modified oligonucleotide using the analog are very rigid and inflexible<sup>[17]</sup>. Furthermore, additional research has proven that at least one kind of LNA-modified antisense oligonucleotide is hepatotoxic<sup>[18]</sup>. After developing the aforementioned modified nucleic acid analogs,

other chemically modified nucleic acids have been designed; for example, 2'-O,4'-C-ethylene bridged nucleic acid (ENA)<sup>[19]</sup>, and unlocked nucleic acid (UNA)<sup>[20]</sup> have been developed (Figure 1). In the case of ENA, Morita *et al* showed that the fact that the 2'-O,4'-C-ethylene linkage restricts the sugar puckering to the N-conformation generates a high binding affinity for a complementary RNA strand (i.e., T<sub>m</sub> value increases by approximately 5°C per modified base) and enhances the resistance to nuclease digestion substantially<sup>[19]</sup>. Meanwhile, UNA is highly flexible due to the lack of C2'-C4' bond present in beta-D-ribofuranose as shown in Figure 1. UNA monomers have proven useful for siRNA in combination with LNA, but rigorous cytotoxicity studies have not been performed<sup>[21]</sup>.

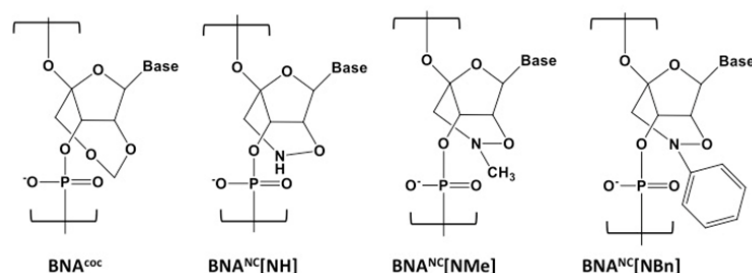
The currently most promising nucleic acid analogs, bridged nucleic acids (BNAs), are molecules that can contain a five-membered or six-membered bridged structure<sup>[15,22-26]</sup>; the comparison of BNA with other nucleic acid analogs is listed in Table 1. After the birth of the 2',4'-BNA (also known as LNA)<sup>[15]</sup>, the structure has evolved through the modification of the linkage connected at the 2',4'-position of the ribose ring. As a result, 3'-amino-2',4'-BNA and 2',4'-BNA<sup>COC</sup> were born as the 2<sup>nd</sup> generation of BNA<sup>[24,25]</sup>. To date, the most updated version of BNA, the 3rd generation of BNA, is 2',4'-BNA<sup>NC</sup> (2'-O,4'-C-aminomethylene bridged nucleic acid), containing a six-membered bridged structure with an N-O linkage<sup>[27]</sup>. Specifically, the new generation 2',4'-BNA<sup>NC</sup> includes 2',4'-BNA<sup>NC</sup>[NH], 2',4'-BNA<sup>NC</sup>[NMe], and 2',4'-BNA<sup>NC</sup>[NBn]<sup>[27]</sup>; the structures of the BNA analogs are shown in figure 2. As for the characteristics of 2',4'-BNA<sup>NC</sup>, when the BNAs are added to DNA or RNA oligonucleotides, the degree of morphological freedom of the oligonucleotides is restricted by its six member methylene bridge structure. Thus, 2',4'-BNA<sup>NC</sup> bases are stacked in the A type conformation by Watson-Crick hybridization, improving hybridization specificity and duplex stability<sup>[27]</sup>. Moreover, the addition of 2',4'-BNA<sup>NC</sup> enhances the T<sub>m</sub> value by 2-9 °C per base<sup>[27]</sup>. The applications using BNA<sup>NC</sup> are discussed as below.

## ANTISENSE AND ANTIGENE APPLICATIONS

Oligonucleotides have been used to govern gene expression as therapeutic tools for biological studies<sup>[28-30]</sup>. Antisense and antigene technologies are excellent examples for therapeutic application, where the combination of natural and modified oligonucleotides that has the ability to form RNA duplexes or triplexes with a great affinity and stability. In antisense technology, the formation of oligonucleotide RNA would block the translational process or cleave the target mRNA with small interfering RNA (siRNA); hence, the level of mRNA expression can decrease significantly. Meanwhile, in



**Figure 1** The structure of DNA, RNA and other nucleic acid analogs. Details on the structures were present in the text.



**Figure 2** The structure of BNA<sup>COC</sup> and other 2',4'-BNA<sup>NC</sup> nucleic acids. For BNA<sup>NC</sup>[NBn], a methylene group exists between N and phenyl.

**Table 1** Comparison of 2',4'-BNANC with other nucleic acid analogs.

Criteria	BNA	LNA	PNA	DNA
Salt conc. for hybridization	Dependent	Dependent	Independent	Dependent
T <sub>m</sub> for each single mismatch <sup>[19,27,22]</sup>	Ca. 4 °C	Ca. 3-4 °C	Lowering 1-5 °C	Lowering 1 °C
Biological stability	Very stable to nuclease	Stable to nuclease	Stable to nuclease and protease	Degradation by nuclease
Thermal stability	Excellent	Good	Good	Moderate
Water solubility <sup>[16]</sup>	Excellent	Good to excellent	Soluble	Soluble
Probe length for diagnostic use	10-25 bases	10-25 bases	13-18 bases	20-30 bases
PCR compatible	Yes	Yes	No	Yes
Body clearance ability <sup>[35]</sup>	Yes	Yes	No	Yes
Inhibition of RNase H	Yes	Less	No	Yes
Hepatotoxicity <sup>[35,18]</sup>	No	Moderate	Moderate	No
Nephrotoxicity <sup>[35,18]</sup>	No	Very low	High	No
Innate immunity stimulation	No	No	Yes	N/A
Gene silencing	Yes	Yes	Yes	N/A
RNA/DNA binding selectivity	Excellent	Good	No preference	N/A
Hybridization affinity with RNA	5-6 °C higher T <sub>m</sub> per base	5-6 °C higher T <sub>m</sub> per base	>1 °C higher T <sub>m</sub> per base	N/A

antigene technology, a formation occurs between a single stranded homopyrimidine triplex-forming oligonucleotide and a homopurine-pyrimidine stretch in target DNA through Hoogsteen hydrogen bonding, where T•A:T and C•G:C base triplets are formed<sup>[31]</sup>. Thus, it is not inconceivable that the triplex formation can inhibit the binding of RNA polymerases in the transcription process, leading to downregulation of target gene expression to a certain extent. To accomplish this purpose, it is imperative that the chemically modified nucleotides bind to their target mRNA or duplex DNA with a great affinity.

Among the chemically modified nucleotides, 2',4'-BNA<sup>NC</sup> nucleotides display improved duplex and triplex affinity due to the reduction of repulsion between the negatively charged backbone phosphodiester groups. Owing to the structural advantage, the hybridization affinity to target RNA molecules has unprecedentedly improved<sup>[32]</sup>. Several lines of evidence indicate that the T<sub>m</sub> value of 2',4'-BNA<sup>NC</sup> bases against complementary RNA enhances +2 to +10 °C per 2',4'-BNA<sup>NC</sup> monomer<sup>[19,27,33,34]</sup>. In addition to the high affinity, incorporation of 2',4'-BNA<sup>NC</sup> bases into oligonucleotides results in the increased resistance to endo- and exonucleases<sup>[27]</sup>. As another example for antisense therapeutics using BNA, BNA-based antisense successfully suppresses hepatic PCSK9 expression that leads to a significant reduction of the serum LDL-C levels of mice, where these findings support the notion that BNA-based antisense oligonucleotides can induce a cholesterol-lowering action in hypercholesterolemic mice<sup>[35]</sup>.

## APTAMER CAPPING APPLICATIONS

Aptamers, oligonucleotides binding to their target molecules with high affinity, are used as research materials in either diagnostic or therapeutic applications. The aptamer oligonucleotides, a library of single-stranded oligonucleotides, usually contain 30 to 60 bases in length using Systematic Evolution of Ligands by EXponential enrichment (SELEX) technique, where the technology initially begins with a random pool of oligonucleotides and finally ends with tightly binding oligonucleotides<sup>[36,37]</sup>. A caveat is that the resulting oligonucleotides are susceptible to nucleases so that the oligonucleotides readily degrade. In order to address this issue, the capping of the ends of aptamers can be used as a solution. The number of enzymes that are able to incorporate modified nucleotides were very limited; however, the process of capping 2',4'-BNA<sup>NC</sup> monomer can be made by a one step enzymatic reaction using 2',4'-bridged nucleoside 5'-triphosphate and terminal deoxynucleotidyl transferase<sup>[38]</sup>. As an example, Kasahara *et al.* reported that the capping of the 3'-end of the thrombin aptamer with 2',4'-BNA<sup>NC</sup> bases significantly enhances the nuclease resistances and binding affinity in human serum<sup>[39]</sup>. In addition to these improvements, the binding ability of the aptamers was not influenced by the capping of 2',4'-BNA<sup>NC</sup> bases<sup>[39]</sup>.

## BNA CLAMP REAL-TIME PCR TECHNOLOGY AND DETECTION ASSAYS

One of the sensitive diagnostic methods for quantitation of changes in gene expression related to cancer or other diseases is the clamp real-time PCR technology<sup>[40]</sup>. The technology can be used with 2',4'-BNA<sup>NC</sup> bases to improve the sensitivity of mutation detection due to the fact that the 2',4'-BNA<sup>NC</sup> bases have the ability to interact with the target nucleic acids with better affinity and stability in comparison with other chemically modified nucleotides. Rahman *et al.* also reported that, compared to other probe technologies,

2',4'-BNA<sup>NC</sup> probes have proven to be a sensitive, accurate and robust detection system<sup>[27]</sup>. Recently, our research group has showed that the results using BNA clamp technology against EGFR T790M mutation was very promising, where the real-time PCR detection displayed the highly selective amplification of the target gene when as little as 0.01% mutated DNA is in the sample analyzed<sup>[41]</sup>. This result reflects that 2',4'-BNA<sup>NC</sup> based probes are ideal for discriminating mutated sequences among wild-type sequences. Similar to the clamp technology, a bead-based suspension assay was developed using 2',4'-BNA<sup>NC</sup> probes, and the assay allows quantitative detection of mutations in the human DNA methyl transferase 3A gene (DNMT3A), whose mutations are found in several hematologic malignancies<sup>[42]</sup>. This assay was shown to be a rapid, easy and reliable method with a sensitivity of 2.5% for different mutant alleles<sup>[42]</sup>. This type of assay can be added to the repertoire of molecular diagnostic techniques with respect to the analysis of biological markers in genomic research. One of the sensitive diagnostic methods for quantitation of changes in gene expression related to cancer or other diseases is the clamp real-time PCR technology<sup>[40]</sup>. The technology can be used with 2',4'-BNA<sup>NC</sup> bases to improve the sensitivity of mutation detection due to the fact that the 2',4'-BNA<sup>NC</sup> bases have the ability to interact with the target nucleic acids with better affinity and stability in comparison with other chemically modified nucleotides. Rahman *et al.* also reported that, compared to other probe technologies, 2',4'-BNA<sup>NC</sup> probes have proven to be a sensitive, accurate and robust detection system<sup>[27]</sup>. Recently, our research group has showed that the results using BNA clamp technology against EGFR T790M mutation was very promising, where the real-time PCR detection displayed the highly selective amplification of the target gene when as little as 0.01% mutated DNA is in the sample analyzed<sup>[41]</sup>. This result reflects that 2',4'-BNA<sup>NC</sup> based probes are ideal for discriminating mutated sequences among wild-type sequences. Similar to the clamp technology, a bead-based suspension assay was developed using 2',4'-BNA<sup>NC</sup> probes, and the assay allows quantitative detection of mutations in the human DNA methyl transferase 3A gene (DNMT3A), whose mutations are found in several hematologic malignancies<sup>[42]</sup>. This assay was shown to be a rapid, easy and reliable method with a sensitivity of 2.5% for different mutant alleles<sup>[42]</sup>. This type of assay can be added to the repertoire of molecular diagnostic techniques with respect to the analysis of biological markers in genomic research.

## CONCLUSION

We have discussed the potential of the new generation BNA. The BNA technology is very promising mainly because of the great affinity against the complementary RNA or DNA and the great cellular nuclease resistance. We can envision that BNA like LNA will be more widely used in antisense oligonucleotide technology, in the field of molecular diagnostics, and in the newly emerging field of siRNAs. In addition, we envision that BNA will be further used as in situ hybridization probes (also known as FISH probes) and single nucleotide polymorphisms discrimination. BNA's clear promise has prompted scientists to produce a diverse set of BNA iterations; for example, amido-BNA, (S)-cEt-BNA, sulfonamide-BNA, benzylidene acetal-type BNA, and guanidine-BNA were recently synthesized. Amido-BNA has a similar effect on antisense knockdown as do other BNAs, and significantly improves cellular uptake as a salient feature<sup>[43]</sup>; (S)-cEt-BNA, a constrained 5-methyluracil nucleoside, can serve as a key gapmer unit in antisense oligonucleosides using relatively short steps in the synthesis process<sup>[44]</sup>; sulfonamide-BNA

enhances nuclease resistance and has a selective effect on the binding affinity towards single-stranded RNA<sup>[45]</sup>; benzylidene acetal-type BNA, a bridged structure that exhibits cleavage by external stimuli, displays different stability and resistance to enzymatic digestion<sup>[46]</sup>; guanidine-BNA, a BNA analog with guanidine attached, significantly improves thermal stability and nuclease resistance and interestingly displays high binding affinity for single-stranded DNA specifically<sup>[47]</sup>. As the efforts to seek better BNA analogs continue, we can easily expect more examples of modified BNA to emerge. Meanwhile, it is imperative that more investigation of these new compounds should be required for practical applications. Taken all together, the technologies using BNA bases have a great potential to become the next generation methods for disease detection and therapy, and BNA will no doubt play a central role as essential molecules in these fields.

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## CONFLICT OF INTERESTS

The Author has no conflicts of interest to declare.

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