Expanding the applications of locked nucleic acids

Rakesh N Veedu* & Jesper Wengel

Nucleic Acid Center, Department of Physics and Chemistry, University of Southern Denmark, Campusvej 55,
Odense M, 5230, Denmark

E-mail: rnv@ifk.sdu.dk

Different LNA nucleoside 5′-triphosphates have been synthesised and their substrate specificities to various DNA and RNA polymerases by performing primer extension reactions, PCR amplification and *in vitro* transcription reactions have been investigated.

Oligonucleotide based drug development has attracted a great deal of interest in recent years, primarily by focusing on gene silencing technologies (antisense, siRNA, antiMIRs *etc.*)[1]. With respect to the Nobel prize awarded for the discovery of RNA interference (RNAi)[2,3] in 2006, this area of research has been widely accepted and has paved the way for launching many new R&D pharma companies around the globe in search of developing new nucleic acid based therapeutics against a wide array of human maladies. Another class of oligonucleotides that is of interest as therapeutics is the so-called aptamers[4-7]. Aptamers are short oligonucleotides which can bind to their target with high affinity and specificity and they are typically identified using an *in vitro* evolution process termed SELEX (Systematic evolution of ligands by exponential enrichment)[8-13]. To exploit the technologies mentioned above, it is inadequate to use oligonucleotides composed of naturally occurring nucleic acids as they pose limitations such as rapid degradation to nucleases and poor binding affinity. To circumvent these problems, a number of nucleic acid analogues have been developed.

Among all nucleic acid analogues reported to date, a very successful non-natural nucleoside is the one called LNA (locked nucleic acid). LNA nucleotide monomers (**Figure 1**) are nucleic acid analogues with a fixed C3′-endo/N-type furanose conformation that mimics the ribose conformation of A-type helical RNA[14-17]. Incorporation of one or more LNA units into a double stranded nucleic acid dramatically increases the melting temperature of the complex.

Hence, for example, LNA allows the use of shorter probes in detection assays and also shorter therapeutic oligonucleotides. Another very important feature of LNA is that it improves the biostability of oligonucleotides, *i.e.* oligonucleotides comprising LNA are less prone to degradation by nucleases[18,19]. The remarkable properties exhibited by LNA has attracted accelerated research globally since the invention has demonstrated unique applicability within numerous areas of chemical biology[17-22]. To further expand the therapeutic application of LNA nucleotides, we envisioned and initiated the development of LNA-modified aptamers by SELEX. In order to apply LNA-modified sequences in SELEX, enzymatic polymerisation of oligonucleotides containing LNA nucleotides has to be efficient. Toward this direction, we have synthesised LNA nucleoside 5′-triphosphates (LNA-NTPs) and investigated its substrate specificities with various DNA and RNA polymerases[23-27].

Initially we investigated the incorporation of LNA-nucleotides in DNA strands by primer extension assays. In such experiments, a short DNA primer oligonucleotide sequence is annealed to a template sequence. Upon incubation the polymerase extends the primer strand by sequential incorporation of the deoxyribonucleotides (dNTPs). The extension products are then analysed by performing polyacrylamide gel electrophoresis followed by phosphor imaging for which the primer sequence is 5′-^{32}P labeled. Our first set of experiments revealed that Phusion™ HF DNA polymerase can incorporate
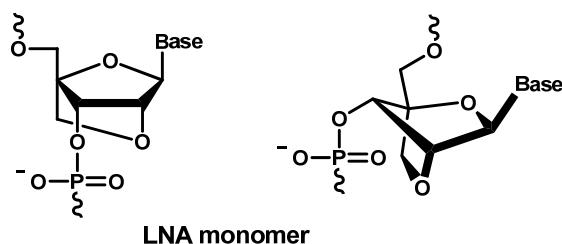
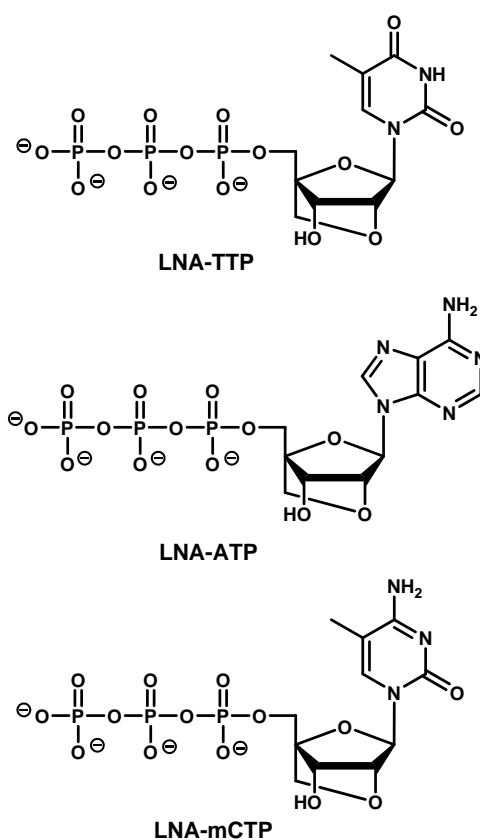**Figure 1** — Structural representation of LNA nucleotide monomers.

Figure 2 — Structural representation of LNA nucleoside 5′-triphosphates.

LNA-T nucleotides opposite to DNA-A nucleotides of the template strand and further extend the strand to full-length[23,24]. Inspired by this finding, we then synthesized LNA-ATP (**Figure 2**) and investigated its substrate properties as well. A full-length product was again achieved after incorporating LNA-A nucleotides at the required positions by Phusion™ HF DNA polymerase[23]. Later on, experiments were conducted to study successive incorporation of LNA-T and LNA-A nucleotides. The results demonstrated

that up to three LNA-T and up to eight LNA-A nucleotides can be incorporated successively[23].

We carried out another experiment in which the template sequence contained two LNA-T and two LNA-A nucleotides along with DNA nucleotides. Phusion™ HF DNA polymerase successfully incorporated natural as well as LNA nucleotides opposite to the LNA nucleotides of the template strand and also afforded the fully extended product[25]. Later we found that 9°N$_m$™ DNA polymerase can also accept LNA-T and LNA-A nucleotides as substrates and also afford full length products as mentioned for Phusion™ HF DNA polymerase[25]. However, we observed product degradation for reactions involving natural dNTPs, whereas this was not an issue in the case of LNA containing reactions.

LNA-5-methyl CTP (LNA-mCTP) (**Figure 2**) was likewise subjected to enzymatic incorporation studies. In addition to Phusion™ HF DNA polymerase, we found that KOD DNA polymerase can efficiently incorporate LNA-mC nucleotides affording the desired full-length products[27]. It was further demonstrated that KOD DNA polymerase can extend the primer strand by incorporating eight consecutive LNA-mC nucleotides[27]. As the next step, an extension experiment was initiated by using a dNTP mix containing LNA-ATP, LNA-TTP, LNA-mCTP and dGTP. In this experiment the primer had to be extended by twelve incorporations of LNA nucleotides (three LNA-Ts, four LNA-mCTPs and five LNA-ATPs). Polyacrylamide gel electrophoresis showed that KOD DNA polymerase efficiently incorporated all the three LNA nucleotides at the required positions and yielded the fully extended product[27]. The extension product was further verified by MALDI-TOF MS analysis.

The results obtained from primer extension assays led us to investigate polymerase chain reaction (PCR) amplification using LNA nucleotides. We used LNA-ATP as the LNA building block and 9°N$_m$™ DNA polymerase or Phusion™ HF DNA polymerase in our initial set of investigations. 9°N$_m$™ DNA polymerase afforded the amplified LNA containing PCR product in good yield whereas Phusion™ HF DNA polymerase afforded the product in only low yield[25]. The products were verified by MALDI-TOF MS analysis. However, it was noted that the conditions optimized for PCR using LNA nucleotides were not suitable for those of natural nucleotides which might be due to their decreased exonuclease resistance. Recently, it was observed that KOD DNA polymerase

Table I — Highlight of LNA nucleotide incorporations

LNA nucleotide incorporations into DNA strands

Polymerases	Incorporations by primer extension assays	PCR
Phusion™ HF DNA polymerase	√	+
9° Nm™ DNA polymerase	√	+++
KOD DNA polymerase	√	+++++

LNA nucleotide incorporations into RNA strands

Polymerase	Incorporations by *in vitro* transcription assays
T7 RNA polymerase	√

afforded the amplified LNA containing PCR product in excellent yield (**Table I**, ref 27). The product was again verified by MALDI-TOF MS analysis.

In the next phase, we investigated LNA nucleotides as substrates for RNA polymerases by performing *in vitro* transcription reactions. In this experiment a short DNA promoter sequence is annealed to a DNA template sequence which upon incubation with the polymerase will lead to the synthesis of a single stranded RNA as the product by incorporation of the ribonucleotides (NTPs). First, we tested the incorporation of LNA-ATP with two incorporation sites in the resulting RNA transcript. Analysis clearly demonstrated that T7 RNA polymerase can accept LNA-ATP as a substrate affording the full-length RNA transcript[25]. Another experiment aiming to evaluate the successive incorporations of LNA-A nucleotides revealed that T7 RNA polymerase can incorporate upto eight LNA-A nucleotides continuously while still yielding the expected full-length RNA transcript (**Table I**, ref. 25). An experiment performed by using an LNA-modified DNA template revealed that T7 RNA polymerase efficiently transcribed and afforded the LNA-incorporated full-length RNA product (ref. 25).

Based on the findings portrayed above, we envision that LNA aptamers can be evolved by conventional SELEX processes.

Acknowledgements

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