Polymerase directed incorporation studies of LNA-G nucleoside 5′-triphosphate and primer extension involving all four LNA nucleotides†

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For the first time, the enzymatic incorporation of LNA-G nucleotides into DNA strands has been investigated. KOD DNA polymerase accepted LNA-GTP as a substrate and provided a full-length dsDNA product modified with LNA-G nucleotides. Most importantly, an extension experiment was conducted using a reaction mixture containing LNA-A, LNA-T, LNA-G and LNA-^{m}C nucleotides as the only NTP sources and this showed that KOD DNA polymerase can even incorporate 21 LNA nucleotides successively to a full-length extension product. Also, the experiments revealed that LNA-containing extension products were much more resistant towards the 3′→5′ exonucleolytic cleavage activity of the enzyme than DNA extension products.

In recent years, nucleic acid based drug development relating to gene silencing technologies such as antisense, siRNA, *etc.*[1–4] has attracted a great deal of interest and formed the basis for launching many new R&D level pharma companies. Another class of nucleic acid based therapeutic candidates is the so-called aptamers[5–8] which are short oligonucleotides that can bind to a specific target with high affinity and specificity because of their ability to form diverse three dimensional structures. Aptamers are generally identified by an *in vitro* evolution process referred to as SELEX (Systematic Evolution of Ligands by EXponential enrichment).[9–14] Aptamers composed exclusively of natural DNA or RNA nucleotides are poorly suitable for many applications *in vitro* and *in vivo* because of their low degree of nucleolytic stability and poor binding affinity. To overcome these limitations, numerous nucleic acid analogs have been introduced over the years, including the prominent and successful locked nucleic acid (LNA) modification.[15–23]

LNA is a nucleic acid analogue that is conformationally restricted with a fixed C3′-endo/N-type furanose conformation that mimics the ribose conformation of A-type helical RNA (Fig. 1).[15–17] Since the invention in the late 1990's, LNA has been used extensively for various applications taking advantage of its remarkable properties such as unprecedented binding affinity to complementary sequences and increased resistance to nucleases compared to the natural nucleotides.[18–23] To exploit further the use of LNA nucleotides in therapeutics, we have envisioned and initiated the development of LNA-modified aptamers by SELEX techniques. However, to apply LNA-modified sequences in SELEX processes, the enzymatic polymerisation of oligonucleotide libraries containing LNA nucleotides has to be efficient. To investigate this, we[24–29] and others[30,31] have synthesised LNA nucleoside 5′-triphosphates (LNA-ATP, LNA-TTP, LNA-^{m}CTP) and investigated their substrate specificities towards various DNA and RNA polymerases. Herein, we report the synthesis and enzymatic incorporation of LNA-G nucleotides into DNA oligonucleotides. More importantly, we demonstrate a primer extension product from using only the four LNA nucleotides, LNA-ATP, LNA-TTP, LNA-^{m}CTP and LNA-GTP (Fig. 1).

Primer extension assays were carried out to investigate the incorporation of LNA nucleotides. In such an experiment, a short primer DNA sequence is annealed to a template sequence, addition of polymerase and nucleoside triphosphates then extends the primer strand by sequential incorporation of the desired nucleotides complementary to those of the template strand. The extension products can be analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) followed by phosphor imaging, when the primer sequence is 5′-end labeled with radioactive phosphorus (^{32}P). First, we

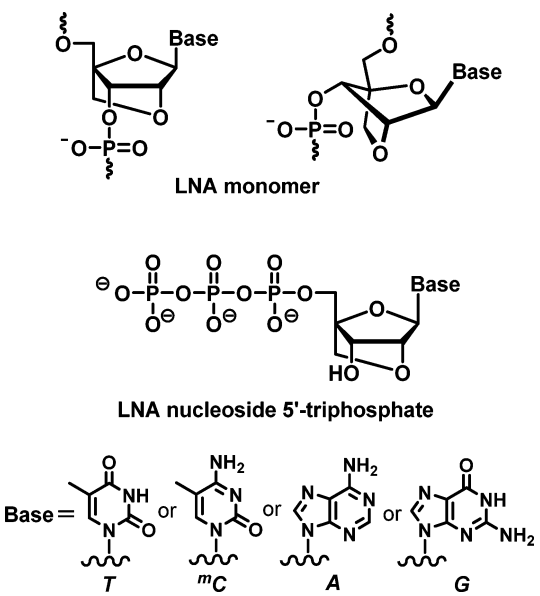

Fig. 1 Structural representation of LNA monomers and LNA nucleoside 5′-triphosphates.

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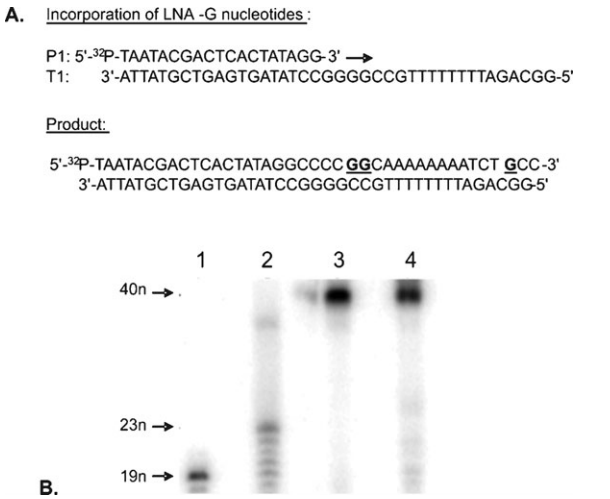

Fig. 2 A. Primer sequence P1, template sequence T1, extension product with LNA-G incorporations (bold and underlined). B. Gel-electrophoresis image of the enzymatic incorporation of LNA-G nucleotides into a DNA strand. Lane 1: primer, lane 2: negative control (dCTP, dATP and TTP), lane 3: positive control (dCTP, dATP, dGTP and TTP), lane 4: LNA-G incorporation (dCTP, dATP, LNA-GTP and TTP).

tested the enzymatic incorporation of LNA-G nucleotides into a DNA strand. The 40n long template sequence, T1 (Fig. 2A), was designed with three sites of incorporations (dC nucleotides in the template strand, complementary to where the LNA-G has to be incorporated) at positions 24, 25 and 38. The design is somewhat challenging as it requires consecutive incorporation of two LNA-G nucleotides. The experiment demonstrated that KOD DNA polymerase can accept LNA-GTP (Fig. 1) as a substrate by incorporating LNA-G nucleotides at the desired positions and also by yielding the expected fully extended product (Fig. 2B). In parallel to the LNA incorporation, we have also performed positive (using DNA nucleotides) and negative (absence of dGTP or LNA-GTP in the mix) control experiments to carefully monitor the accuracy of LNA incorporations.

As we have confirmed the enzymatic recognition of all four LNA-nucleoside 5′-triphosphates (the results described above plus the results in our previous reports[24–29]), an extension experiment was performed with all four LNA nucleotides (Fig. 1). In this experiment the primer DNA strand has to be extended only with LNA nucleotides, *i.e.* the 40n long template DNA sequence T2 (Fig. 3A) directs the incorporation of nine LNA-G nucleotides, five LNA-A nucleotides, four LNA-^{m}C nucleotides and three LNA-T nucleotides totaling 21 LNA nucleotides to be incorporated successively. Surprisingly, the experiment showed that KOD DNA polymerase can efficiently incorporate LNA nucleotides exclusively to yield the expected full-length extension product (Fig. 3B). However, relatively longer extension time (120 min) was required to obtain the full-length product in a high yield whereas the DNA nucleotide based reaction product was obtained within 3 min incubation. Furthermore, we observed that the LNA-containing product was remarkably stable towards the 3′→5′ exonuclease activity of the enzyme even after 120 min of incubation at 74 °C, whereas the natural nucleotide

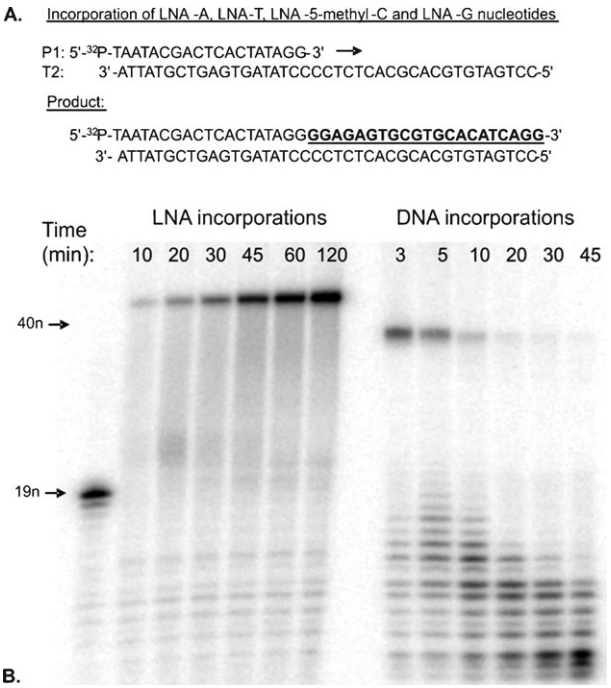

Fig. 3 A. Primer sequence P1, template sequence T2, and the extension product with LNA and DNA nucleotide incorporations (bold and underlined). B. Gel-electrophoresis image of the enzymatic incorporation of LNA nucleotides and DNA nucleotides. LNA incorporations are followed from 10 min to 120 min of incubation and DNA incorporations are from 3 min to 45 min of incubation at 74 °C.

product was found to be partially degraded after only 3 min of reaction (Fig. 3B). We observed a slower migration of the LNA containing product band compared to that of the DNA nucleotide product in accordance with our previous observations.[29] The LNA extension product was further verified by performing MALDI-TOF MS analysis (Fig. 4).

In summary, we have demonstrated that LNA-G nucleotides can be incorporated into a DNA strand by KOD DNA polymerase. More interestingly, it has been shown that KOD

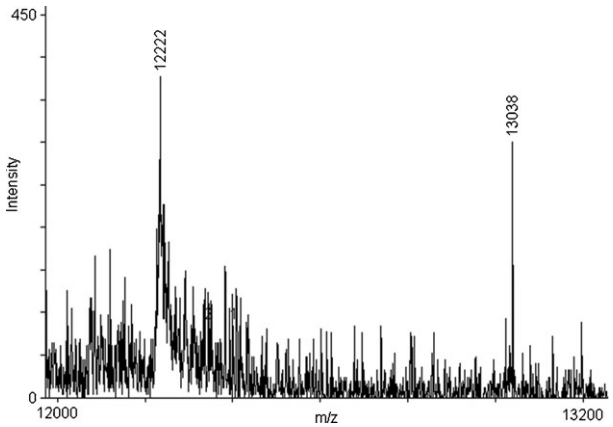

Fig. 4 MALDI-TOF MS analysis of the primer extension products using all four LNA nucleotides. Calculated mass for the top strand with 21 LNA nucleotide incorporations, 13 038 Da; signal observed, 13 038 Da. Calculated mass for the bottom template strand, 12 198 + Na^+ (12 221 Da); signal observed, 12 222 Da.

DNA polymerase can successively incorporate up to 21 LNA nucleotides from a mixture containing LNA-ATP, LNA-TTP, LNA-GTP and LNA-^{m}CTP. These findings open new applications of LNA nucleotides in biological research in general wherever the use of LNA nucleotides can be beneficial.

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Experimental

Materials and methods

LNA-GTP was synthesized according to our previous report for LNA-triphosphate synthesis.[25] The primer and template oligonucleotide sequences were purchased from Sigma-Genosys, Brøndby, Denmark. [γ-^{32}P]-ATP ($\sim$6000 Ci mmol^{-1}) was purchased from GE Healthcare, Hillerød, Denmark. T4 polynucleotide kinase was purchased from New England Biolabs (supplied by Medinova, Denmark). KOD DNA polymerase was purchased from TOYOBO, Japan (supplied by Novagen, Germany). MALDI-TOF MS analysis was performed with a Voyager Elite (PerSeptive Biosystems) MALDI-TOF apparatus recording the spectra in positive ion mode using delayed ion extraction.

General procedure for primer extension reactions

The primer sequences were 5′-^{32}P labelled by [γ-^{32}P]-ATP using T4 polynucleotide kinase following manufacture's recommendations. The 5′-end labelled primers were annealed to the templates by mixing primer and template in a molar ratio of 1 : 1 in a transcription optimized 5× buffer (from Promega, containing 40 mM Tris-HCl (Ph 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) and heating to 82 °C for 3 min, followed by slow cooling to room temperature. The reaction mixtures were prepared in a total volume of 20 μL by adding 2.5 μL of 10× KOD DNA polymerase buffer (included in the KOD DNA polymerase kit), 1 μL of $MgCl_2$ (25 mM), 0.5 μL of $MnCl_2$ (100 mM), 1.5 μL dNTPs–LNA-NTPs mixture (2.5 mM), 13.3 μL of two times distilled water, 0.6 μL of a solution containing 5′-^{32}P-labelled primer-template complex (50 pmol μL^{-1}) and 0.6 μL of KOD DNA polymerase (2.5 U μL^{-1}). The reaction mixtures were gently vortexed and incubated for 30 min at 74 °C for the reactions involving LNA-GTP. Positive and negative control reactions were stopped after 3 min incubation at 74 °C as prolonged incubation resulted in product degradation. In the extension experiment using all LNA nucleotides and all DNA nucleotides, 3 μL of the samples were taken at different time intervals (for LNA: 10, 20, 30, 45, 60 and 120 min; and for DNA: 3, 5, 10, 20, 30, 45 min). The polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA). Analysis of the products was performed by gel electrophoresis on a 13% 7 M urea polyacrylamide gel in the presence of a TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA) of pH 8.4 followed by phosphor imaging.

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