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Enzymatic polymerisation involving 2′-amino-LNA nucleotides

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ABSTRACT

The triphosphate of the thymine derivative of 2′-amino-LNA (2′-amino-LNA-TTP) was synthesised and found to be a good substrate for Phusion® HF DNA polymerase, allowing enzymatic synthesis of modified DNA encoded by an unmodified template. To complement this, 2′-amino-LNA-T phosphoramidites were incorporated into DNA oligonucleotides which were used as templates for enzymatic synthesis of unmodified DNA using either KOD, KOD XL or Phusion polymerases. 2′-Amino-LNA-T in the template and 2′-amino-LNA-TTP as a substrate both decreased reaction rate and yield compared to unmodified DNA, especially for sequences with multiple 2′-amino-LNA-T nucleotides.

The use of oligonucleotides (ONs) for such applications as drugs or diagnostic probes is appealing but often requires ONs that are structurally altered from natural ONs in order to improve biostability, pharmacokinetics and function.[1–5] One example is the aptamer Macugen (Pegaptanib). Aptamers are single stranded ONs that are folded to form a stable three-dimensional structure capable of specifically binding a target molecule with high affinity.[6,7] Macugen is modified with an inverted nucleotide at the 3′-end, 2′-OMe-RNA purine nucleotides, and 2′-F-DNA pyrimidine nucleotides and conjugated to a 40 kDa PEG to improve biodistribution.[8,9]

With respect to both stability and function, the conformationally locked nucleotide modification 2′-amino-LNA[10–13] (2′-amino locked nucleic acid) has interesting characteristics. 2′-Amino-LNA monomers thus protect antisense gapmer ONs from degradation by nucleases,[14] they increase duplex stability which may improve target affinity and specificity, and the 2′-nitrogen may serve as a handle for attachment of functional groups.[11,15–19] As an example, N2′-pyrene-functionalised 2′-amino-LNA monomers have been used in the design of probes suitable for signalling of hybridisation events.[20,21]

Aptamers can be evolved by a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment). SELEX is an elegant solution to the inherent difficulty in selection of individual components from large libraries of oligonucleotides.[4,6,7] It has previously been found that when 2′-amino-LNA is incorporated into an avidin aptamer, the binding affinity towards avidin is not affected significantly.[22] However, this study refers to aptamer modification after initial identification of the unmodified DNA aptamers by SELEX, only few amino-LNA incorporation sites were studied, and it is possible that a more systematic study[15,23] would reveal optimal incorporation sites.

SELEX in the context of aptamers relies on PCR amplification of target binders after each round of selection, gradually increasing the proportion of active species in the library. To improve functional oligonucleotide characteristics, one approach is working from a library of chemically modified oligonucleotides.[24,25] Direct amplification of a chemically modified pool for the next round of selection can be difficult, so it can be necessary to add intermediate steps, such as transcription of modified oligonucleotides into natural DNA, which can then be amplified by PCR and subsequently transcribed back into modified ONs through primer extension using modified dNTPs. Various bicyclic and other sugar-modified nucleosides have previously been triphosphorylated and used as substrates for polymerases, including cyclohexenyl nucleic acids,[26] LNA,[27–29] and several other 2′,4′-linked nucleotide analogues.[30,31] While these modifications are allowed both in the template strand and as substrates in enzymatic polymerisation reactions, they may decrease the efficiency of the reactions compared to reactions with natural substrates and templates, and limits on their positioning and number of incorporations may exist. With respect to LNA, KOD and Phusion® HF DNA polymerases have proven among the most successful enzymes.

We herein present the first results on DNA-dependent 2′-amino-LNA polymerisation and 2′-amino-LNA-dependent DNA polymerisation. We investigated primer extension from templates

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modified with 2′-amino-LNA-T to give natural DNA as well as primer extension with DNA templates with modified 2′-amino-LNA-TTP as a substrate.

2′-Amino-LNA-thymine triphosphate **4** was synthesised from the DMT protected nucleoside **1**.[11] One potential route would involve deprotection of the 5′-hydroxy group followed by phosphorylation with $POCl_3$, treatment with tri-*n*-butylammonium pyrophosphate, and neutral hydrolysis, corresponding to what we earlier used for preparation of the triphosphorylation of LNA.[27] Here, however, we employed a slight modification of the method described by Ludwig and Eckstein.[32,33] As shown (Scheme 1), nucleoside **1**[11] was first acetylated at the 3′-hydroxy group by reaction with acetic anhydride followed by deprotection of the 5′-hydroxy group by reaction with dichloroacetic acid and triethylsilane. The resultant nucleoside was then reacted with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one followed by a double displacement reaction with pyrophosphate to yield a cyclic intermediate which is first oxidised and then hydrolysed. Removal of the 2′- and 3′-protecting groups under basic conditions eventually yielded the triphosphate **4** in an overall yield of 5% from **1** (see Supplementary data for details).

Appropriate primers and templates were designed, and template strands modified with 2′-amino-LNA-T monomers were synthesised by automated DNA synthesis using known 2′-amino-LNA-T phosphoramidite with the N2′-functionality protected by trifluoroacetylation. Phosphoramidites and modified ONs were synthesised as previously described.[11,34]

We first tested the enzymatic synthesis of an unmodified DNA strand by Phusion polymerase using a modified template (T1.1) which contains LNA-A at positions 31 and 33 and 2′-amino-LNA-T at positions 36 and 39 (see Supplementary data for details). As seen in Figure 1, full length product (43n) was dominant after 10 min reaction. Pausing bands are observed corresponding to the first four–five nucleotides, possibly due to poor extension and/or premature termination of the primer past the initial all-G sequence, and at positions 37n (after 1st incorporation), 40n (after 2nd incorporation), 42n and 43n. When the reaction time was extended much beyond 10 min, the (all-DNA) product began to be degraded likely because of the exonuclease activity of the enzyme. As bands are also seen below the 19n band representing the primer, some of the bands representing less than full length product may be due to degradation products. In the negative control, a few bands were seen indicating that the growing ON product strand can be extended past the first amino-LNA-T, but not the second. This points to some promiscuity in the system, but importantly no full length product was observed.

In the reaction depicted in Figure 1, the product was isolated from the reaction mixture by phenol-chloroform extraction followed by precipitation from ethanol. The isolated product was redissolved in H_2O (half the original reaction volume), and 1 μL of this solution was added to 2 μL of a formamide loading solution and denatured by heating prior to loading onto a gel. Using this template, if less denaturing conditions are used (such as a lower proportion of loading solution to reaction sample, or if the phenol-chloroform extraction is omitted) a band appeared above the full length product in the gel, that is, representing a species with decreased gel mobility compared to the 43n product (Figs. S3.1 and S3.2, Supplementary data). We hypothesise that this band represents non-denatured double stranded reaction product. Due to the presence of two LNA-A's, two LNA-T's, and a high GC content, the stability of the resulting duplex is likely to be very high and the duplex difficult to denature, especially in the presence of salts, buffers, polymerase, and excess dNTPs. Other possibilities are that this band represents an oligonucleotide–polymerase complex, or some tertiary structure such as a G-quadruplex.

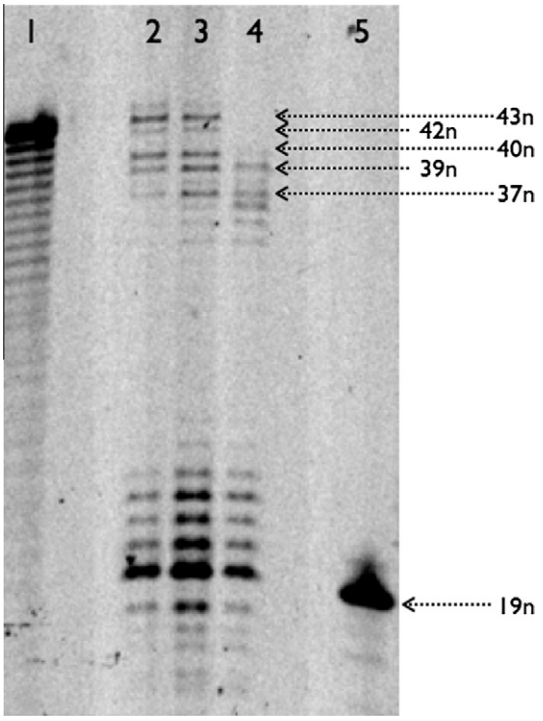
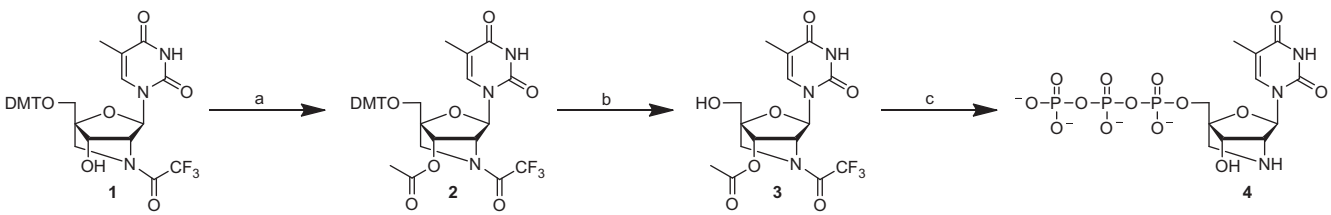


Figure 1. Enzymatic extension using Phusion polymerase of primer P1.2 annealed to modified template T1.1 (43n) by Phusion or KOD DNA polymerase. **1**: 43n FAM labelled DNA marker M1.2; **2**: extension reaction, dATP, dTTP, dCTP, dGTP (10 min); **3**: extension reaction, dATP, dTTP, dCTP, dGTP (5 min); **4**: negative control reaction, dTTP, dCTP, dGTP (10 min); **5**: primer P1.2.

When extension reactions were performed using template T2.1 (Table 1), a 44n template with three incorporated 2′-amino-LNA-T nucleotides at positions 29, 35, and 41, non-denatured double stranded product was not seen, even when the samples were loaded directly from the reaction mixture without phenol-chloroform extraction. This points toward the effect described above being template-specific. Phusion polymerases gave full length product in the case of reaction with all four dNTPs, as well as a (44+1)n product. The negative control showed that almost all extension was halted opposite to the first incorporation site (29n) or one nucleotide past



Scheme 1. Reagents and conditions: (a) $(CH_3CO)_2O$, pyridine, CH_2Cl_2, rt, 17 h. (b) $CHCl_2CO_2H$, $(CH_3CH_2)_3SiH$, CH_2Cl_2, rt, 3 h (60% over two steps). (c) (i) 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, dioxane, pyridine (ii) $(HNBu_3)_2H_2P_2O_7$, DMF, Bu_3N (iii) I_2, pyridine, H_2O (iv) Na_2SO_3 (aq) (v) H_2O, NH_3 (aq) (8%).

Table 1
Sequences of templates (T1.1–T4.1), primers (P1.1–P3.1), and markers (M1.1–2.1) used in primer extension reactions

Name (length)	Sequence
T1.1 (43)	3′-ATTATGCTGAGTGATATCCGGGGCCGACCCA^{L}CA^{L}CC$\underline{T}$GG$\underline{T}$CTGG-5′
T2.1 (44)	3′-ATTATGCTGAGTGATATCCGAACAGCCA$\underline{T}$ACAGG$\underline{T}$GAAAG$\underline{T}$CGG-5′
T3.1 (35)	3′-TAAATCCACTGTGATATCTTCCTCGCTAGCGTCTC-5′
T4.1 (44)	3′-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5′
P1.1 (19)	5′-(^{32}P)-TAATACGACTCACTATAGG-3′
P1.2 (19)	5′-(FAM)-TAATACGACTCACTATAGG-3′
P3.1 (21)	5′-(Cy5)-ATTTAGGTGACACTATAGAAG-3′
M1.1 (43)	5′-(^{32}P)-TAATACGACTCACTATAGGCCCCGGCTGGGTGTGGACCAGACC-3′
M1.2 (43)	5′-(FAM)-TAATACGACTCACTATAGGCCCCGGCTGGGTGTGGACCAGACC-3′
M2.1 (44)	5′-(FAM)-TAATACGACTCACTATAGGCTTGTCGGTATGTCCACTTTCAGCC-3′

$\underline{T}$ = Amino-LNA-T monomer, A^{L} = LNA-A monomer, FAM = fluorescein.

it as only very faint bands are seen at >29n. Bands were also seen at 1nt intervals from 14nt to 25nt in both the reaction and the negative control. As described above, it is not clear whether these bands might be due to degradation or, at least partly, to shorter extension products (Fig. 2).

Primer extension could also be carried out using KOD or KOD XL DNA polymerase. In both cases, the negative control continued past 40n for T1.1 (Fig. S3.2, Supplementary data), and when using T2.1 with KOD XL, full length extension past the modified nucleotides in the template required such error-prone conditions that full length extension was also seen in the negative control reaction (Fig. S3.5, Supplementary data).

We also investigated the enzymatic incorporation of 2′-amino-LNA-T into a DNA mixmer ON. An all DNA 35nt template with a single incorporation site opposite to position 28 was used with Phusion polymerase under the same conditions as were used for reading 2′-amino-LNA-modified templates. The primer was extended to full length product within five minutes (Fig. 3, Fig. S3.4, Supplementary data). The negative control stops at 27nt. Degradation products smaller than the primer are seen in both the negative and positive controls even after <1 min (Fig. 3). After 1 h, the degradation products were the only bands seen. The modified product was also degraded, however, in this case degradation largely stopped at position 29, showing that the 3′-5′-exonuclease activity of the polymerase may degrade the modified ON but it cannot continue past the modified nucleotide (Fig. S3.4, Supplementary data). This supports an earlier study showing that 2′-amino-LNA nucleotides protect against nucleolytic degradation.[14]

Next, we performed another extension experiment using a 44n all-DNA template (T4.1, Table 1), which encodes for incorporation of 2′-amino-LNA-T opposite positions 37, 39, and 41. Phusion DNA polymerase efficiently afforded the full length extension product after 120 min reaction (Fig. 4).

No difference in gel migration was observed between the all-DNA and amino-LNA incorporated products (Fig. 4, lanes 4 and 3, Fig. 3, lanes 2 and 3) products. The faintness of the bands in the control reaction may be due to the degradation of the control DNA product.

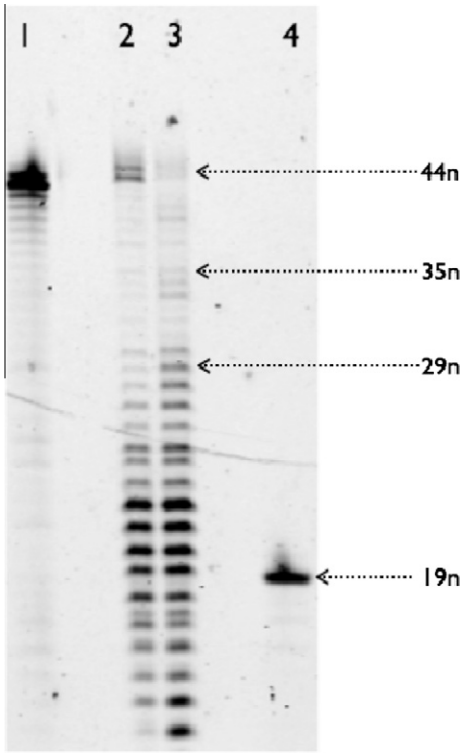


Figure 2. Enzymatic extension using Phusion polymerase of primer P1.2 annealed to modified template T2.1 (44n). **1**: 44n FAM labelled marker M2.1. **2**: Extension reaction, dATP, dTTP, dCTP, dGTP (10 min). **3**: Negative control reaction, dTTP, dCTP, dGTP (10 min). **4**: Primer P1.2.

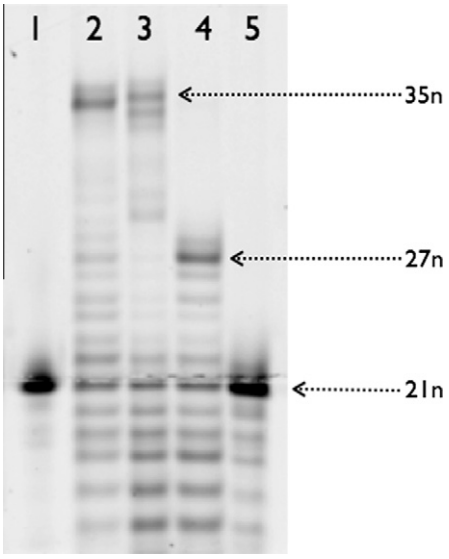


Figure 3. Enzymatic extension using Phusion polymerase of primer P3.1 annealed to template T3.1 (35n). **1**: Primer 3.1. **2**: Positive control, dTTP, dATP,dCTP, dGTP (30s). **3**: Extension reaction, 2′-amino-LNA-TTP, dATP,dCTP, dGTP (10 min). **4**: Negative control reaction, dATP, dCTP, dGTP (30s). **5**: Negative control reaction II, no dNTPs (30s).

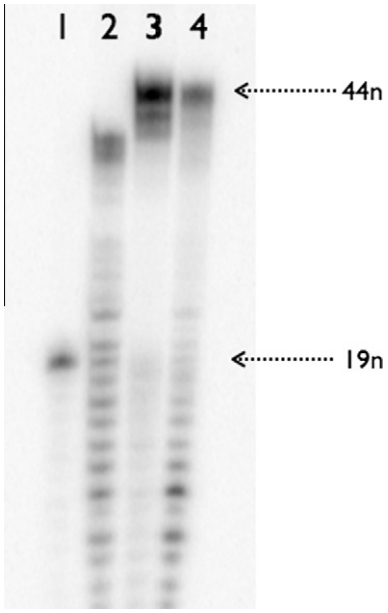


Figure 4. Enzymatic extension using Phusion polymerase of primer P1.1 annealed to template T4.1. **1**: Primer P1.1. **2**: Negative control reaction, dATP, dCTP, dGTP (120 min). **3**: Extension reaction, amino-LNA-TTP, dATP, dCTP, dGTP (120 min). **4**: Positive control reaction, dTTP, dATP, dCTP, dGTP (120 min).

A time-course experiment was carried out for the P1.1/T4.1 system shown in Figure 4. The reaction with 2′-amino-LNA-TTP is much slower than that with dTTP, especially for incorporation of the second and third modified nucleotide, and full length (44n) product does not appear as more than a faint trace until the 60 min time point (Fig. 5). These results show that 2′-amino-LNA can be incorporated into DNA oligonucleotides using Phusion polymerase, but not as well or as quickly as natural nucleotides.

To avoid problems due to degradation, we also attempted DNA-dependent 2′-amino-LNA polymerisation using T3.1 and KOD XL polymerase, which has previously been successful in the case of LNA triphosphates.[29,30] Using this template, we could not,

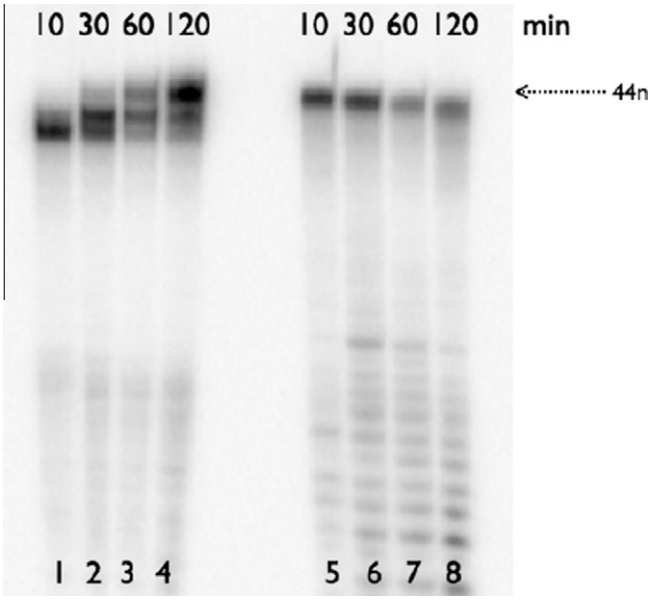


Figure 5. Enzymatic extension using Phusion polymerase of primer P1.1 annealed to modified template T4.1, time-course reaction. **1**–**4**: Extension reaction, 2′-amino-LNA-TTP, dATP, dCTP, dGTP, time points as shown at the top of the gel. **5**–**8**: Positive control reaction, dTTP, dATP, dCTP, dGTP, time points as shown at the top of the gel.

Table 2
Summary of results

Enzyme	Synthesis of DNA from template containing 2′-amino-LNA	Synthesis of ON containing 2′-amino-LNA from DNA template
Phusion	Yes	Yes
KOD	Yes[a]	n.d.
KOD XL	Yes[b]	Yes[c]

[a] Partial extension of negative control.
[b] Partial (T1.1) or full (T2.1) extension of negative control.
[c] Full extension of negative control. n.d. = not determined.

however, find conditions where the primer could be extended to full length without also seeing full length product in the negative control.

Kuwahara et al. have previously investigated the enzymatic incorporation of very similar cyclic nucleotide analogues. Their research showed that bulkiness negatively influenced the incorporation of the modified triphosphates. They also found that incorporation of modifications was more difficult than reading a modified template.[30] We also attempted reading of templates modified with 2′-*N*-glycyl-amino-LNA-T. However, with the conditions and polymerases used, primers could not be extended past the first incorporation site encoded by this modified nucleotide. Polymerisation of N2′-functionalized 2′-amino-LNA nucleotides was tested using triphosphates modified with the less bulky N-substituents methyl and acetyl, but no full length product was observed.

In conclusion, we have successfully synthesised the 5′-triphosphate derivative of the 2′-amino-LNA-T nucleoside as the triethylammonium salt. This modified nucleotide was incorporated into DNA strands by enzymatic synthesis with a rate depending on the sequence and the number of incorporations. Templates chemically synthesised to contain 2′-amino-LNA-T nucleotides are able to efficiently direct enzymatic synthesis of DNA oligonucleotides. Of the polymerases assayed, Phusion is the most suitable, as it provides full length product without strong nuclease activity, and does not extend the negative control to full length (Table 2). However, the efficiency and ease of analysis of the reaction is template dependent and both product degradation and partial extension of the negative control remain challenging.

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Supplementary data

Supplementary data (experimental procedure for synthesis of 2′-amino-LNA-TTP, NMR spectra, additional gel images, and general protocols for primer extension reactions) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.073.

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