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Stepping towards highly flexible aptamers: enzymatic recognition studies of unlocked nucleic acid nucleotides†

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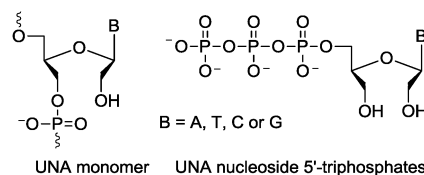
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Enzymatic recognition of unlocked nucleic acid (UNA) nucleotides was successfully accomplished. Terminator DNA polymerase was found to be an efficient enzyme in primer extension reactions. Polymerase chain reaction (PCR) amplification of a 81 mer UNA-modified DNA library was efficiently achieved by KOD DNA polymerase.

Development of nucleic acid therapeutics has attracted significant interest for the treatment of many diseases. With one aptamer-based drug, Macugen (Pegaptanib sodium),¹ on the market for the treatment of age-related macular degeneration (AMD), this class of nucleic acid constructs are an emerging attractive class of therapeutic molecules.² Highly specific DNA or RNA aptamers with high binding affinity to their targets are normally selected from a large pool of oligonucleotides by *in vitro* selection processes.³ Chemically modified aptamers are used to improve pharmacodynamic, as well as pharmacokinetic properties. The application of modified nucleotides in the aptamer selection processes is rather limited due to their poor substrate specificities to polymerases. However, there are a few reports that describe the selection of aptamers in a single step with only one enzymatic protocol that involves polymerase chain reaction (PCR) amplification.⁴ Establishing an efficient PCR method for a library containing modified nucleotides is a key step prior to successful selection of chemically modified aptamers.

Unlocked nucleic acid (UNA) is an RNA mimic in which the bond between the C2' and C3' atoms of the ribose ring is cleaved, which results in high flexibility relative to the parent RNA monomer (Scheme 1).⁵ Thermodynamic stability of i-motif structures can be modulated by introducing UNA nucleotide monomers.⁶ Furthermore, UNA was found to be a useful modification in siRNA-based gene-silencing technology as it can protect the siRNAs from serum degradation and offer



Scheme 1 Structure of UNA nucleotide monomers and triphosphates.

reduced off-target effects while retaining gene silencing potency.⁷ Most importantly, the applicability of UNA nucleotides in aptamer development was recently studied by systematically incorporating them in a known DNA aptamer against thrombin, whereby it was demonstrated that introduction of UNA monomers at specific positions significantly increased the binding affinity.⁸ These remarkable findings clearly substantiate the need to develop UNA-modified aptamers from a large library by *in vitro* selection processes. Herein, for the first time, we report enzymatic recognition studies involving UNA nucleotides.

We investigated UNA nucleotides as substrates for DNA polymerases in line with our current focus on one-step selection of UNA-modified DNA aptamers. First, we synthesized UNA-C nucleoside 5'-triphosphates in small scale according to a procedure published for nucleoside triphosphate synthesis,⁹ after which all four UNA triphosphates (Fig. 1) were prepared in larger scale. Primer extension assays were carried out to screen six different DNA polymerases from both A- and B-families, including Terminator, KOD, Phusion, Pfu, Klenow, and Taq DNA polymerases. Initially, we tested the ability of the polymerases to read UNA nucleotides included in a template strand. Experiments were conducted using only one DNA nucleoside triphosphate, to allow successive incorporation of a single nucleotide directed by the complementary UNA base of the template strand. Chimeric DNA/UNA templates were constructed with a primer binding site followed by seven consecutive UNA nucleotides (Fig. 1a). Reactions were monitored at one, two, five and ten minutes of incubation with each polymerase. Primer DNA was labelled with FAM at the 5'-position for product analysis.

The experiments revealed that Terminator DNA polymerase was the best among the studied polymerases to read through UNA nucleotides and incorporate the complementary DNA nucleotide. Although full-length extension was not observed, in the case of template T1 and T2, Terminator DNA polymerase

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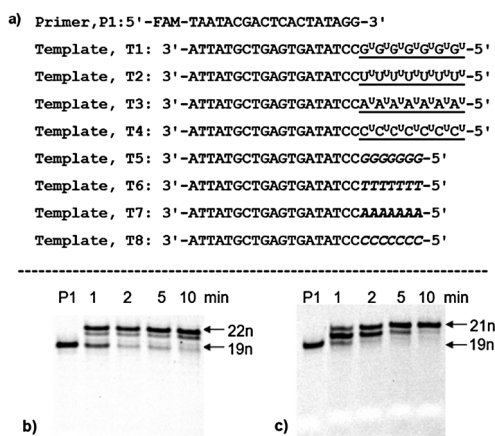


Fig. 1 Primer extension reactions using UNA-templates and UNA-nucleoside 5'-triphosphates. (a) Primer and template sequences. (b) Reading of UNA-G template T1 to incorporate dC by Therminator DNA polymerase. (c) Incorporation of UNA-C nucleotide on T5 by Therminator DNA polymerase. UNA nucleotides are underlined and denoted by a letter 'U' as superscript. Sites for UNA incorporation are italicized.

successfully incorporated up to three nucleotides with good yield (Fig. 1b and Fig. S2b, Supplementary Information†). It was found that ten minutes of incubation was optimal although products were observed even after one minute. Template T3, where dT nucleotide incorporation was tested, yielded only two incorporations (Fig. S3b, Supplementary Information†). Surprisingly, in the case of template T4, we observed partial full-length extension at two minutes of incubation, however, at five and ten minutes, the polymerase generated longer reaction products (Fig. S4b, Supplementary Information†). We suggest that extension of a primer beyond three nucleotides on the UNA-modified template results in a progressive loss of contacts between the polymerase and the template strand as the polymerase translocates into the UNA region of the template, resulting in non-templated nucleotide addition.^{10a} Except, *Pfu*, all other DNA polymerases could add at least one nucleotide (Fig. S1–S4, Supplementary Information†) in the case of templates (T1, T2, T4) containing UNA-G, -U and -C nucleotides, whereas reading a UNA-A-modified template T3 was only accomplished by Therminator, Phusion and Klenow DNA polymerases.

Next, we investigated UNA-nucleoside 5'-triphosphates as substrates for the above mentioned polymerases. In these experiments we used templates T5–T8 (Fig. 1a) containing seven consecutive dG, dT, dA and dC nucleotides, respectively, each of which was designed to encode the complementary UNA-nucleotide. The results again showed that Therminator DNA polymerase was the best at accepting UNA-NTPs, compared to other DNA polymerases. Two consecutive UNA-nucleotide incorporations were observed with templates T5 (Fig. 1c) and T8 (Fig. S8b, Supplementary Information†). In the case of templates T6 and T7, only one UNA nucleotide incorporation was achieved (Fig. S6b and S7b, Supplementary Information†). Except Therminator, all the other polymerases tested failed to incorporate a UNA-A nucleotide when using template T6. Notably, Klenow DNA polymerase was also able to add two UNA-C and -G nucleotides on templates T5 and T8 (Fig. S5d and S8e, Supplementary Information†).

Phusion and KOD DNA polymerases could also incorporate one UNA nucleotide (Fig. S5–S8, Supplementary Information†) whereas *Pfu* and *Taq* DNA polymerases were unable to incorporate even a single UNA nucleotide.

We then carried out experiments to check the fidelity of polymerases when reading and incorporating UNA nucleotides. In these studies, we used Therminator DNA polymerase as it was found to be the best at recognizing UNA nucleotides. To check the incorporation of dC opposite to UNA-G using template T1, we replaced dCTP with dGTP and followed the same experimental procedure as for the one involving dCTP. The result showed no incorporation (Fig. S9b, Supplementary Information†), indicating that the enzyme followed correct Watson–Crick base-pairing rules under the conditions employed. Likewise, fidelity experiments were conducted for other nucleotides using templates T2–T4 and the results again showed no incorporation of the wrong nucleotide (Fig. S9c–e, Supplementary Information†). Next, the fidelity of UNA nucleotide incorporations using Therminator DNA polymerase was conducted in which the correct UNA triphosphate was replaced with the one similar to its complementary nucleotide on the template strand. We used templates T5 and T8 for this analysis, as we observed two UNA nucleotide incorporations on these templates. The results again showed no incorporation of the incorrect nucleotides (Fig. S10b and c, Supplementary Information†). Experiments were also conducted for UNA nucleotide incorporation opposite to UNA modifications on the DNA template. But the polymerases failed to furnish any incorporation.

In all polymerisation reactions discussed above, $MnCl_2$ was supplemented, since Mn^{2+} ions are known to relax the specificity of many DNA polymerases.^{10b} However, experiments were also performed without supplementing $MnCl_2$. For this analysis, we selected template T1 (UNA reading) and template T5 (UNA incorporation) using Therminator DNA polymerase. The expected products were obtained in slightly reduced yields compared to the reactions containing $MnCl_2$ (Fig. S11b and c, Supplementary Information†) by Therminator DNA polymerase. In addition, we also conducted experiments without the presence of any triphosphate as a negative control aimed to check possible dNTP contamination in the reaction mixtures. In all experiments, there was no extension as expected (Fig. S12b and c, Supplementary Information†).

PCR amplification of selected UNA-modified DNA library members is a key step involved in the one-step aptamer selection protocol. Towards this goal, we designed UNA-G modified templates T10 and T11 along with an unmodified template T9 (Fig. 2a) for performing PCR amplification reactions. The reason for using UNA-G nucleotide-modified templates was that the reading and incorporation experiments suggested that UNA-G nucleotide was tolerated best by the enzymes. The results demonstrated that KOD DNA polymerase efficiently amplified full-length all-DNA PCR products in very good yield for template T11 containing two UNA-G nucleotides, however, in the case of template T10 containing one UNA-G nucleotide the product was obtained in lower yield (Fig. 2c). This might be due to the conformational changes imposed on the DNA template by the incorporated UNA nucleotide, making it difficult for the polymerase to read

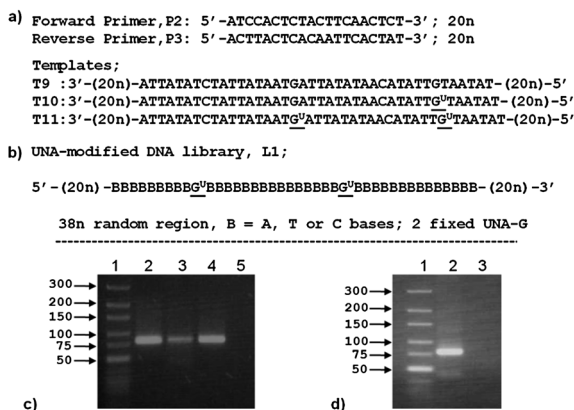


Fig. 2 PCR amplification using UNA-modified DNA template and UNA-modified library. (a) Primer and template sequences; (b) UNA-modified DNA library construct; (c) Agarose gel electrophoresis image of PCR using templates T9–T11, lane 1: DNA size markers in base-pairs, lane 2: PCR using T9, lane 3: PCR using T10, lane 4: PCR using T11, lane 5: PCR without using a template (negative control); (d) Agarose gel electrophoresis image of PCR using library L1, lane 1: DNA size markers in base-pairs, lane 2: PCR using the UNA-modified DNA library, L1, lane 3: PCR without using the library (negative control). UNA nucleotides are underlined and denoted by a letter 'U' as superscript.

and synthesize the complementary strand in the first cycle where it converts UNA/DNA to all-DNA.

Introduction of an additional UNA-G nucleotide, separated by 14 DNA nucleotides from the first one, (template T11) might favor conformational changes needed for enzymatic polymerization. One-step selection of aptamers containing modified nucleotides followed by PCR amplification requires fidelity in the polymerization reactions, to faithfully maintain the functional sequences. The fidelity of PCR based on a UNA-containing template T11 was verified by cloning and subsequent DNA sequencing of the PCR product. The sequencing chromatograms were fully matched the expected PCR product sequence (Fig. S13, Supplementary Information†). We selected fifty clones for this analysis and found the same products in all the cases. In addition to KOD DNA polymerase, Terminator and Phusion DNA polymerases also afforded the expected PCR product but in lower yields although Terminator DNA polymerase was not designed for use in PCR (Fig. S14 a and b, Supplementary Information†).

Based on the results of UNA template-based PCR, we constructed a UNA-containing DNA library L1 (Fig. 2b) towards the one-step selection of UNA aptamers. PCR amplification was conducted using similar experimental protocols as mentioned above for specific enzymes with elevated annealing temperatures (annealing temperature had to be increased by +3 °C as the library L1-based PCR was not optimal at the conditions used for templates T9–T11). KOD DNA polymerase efficiently amplified full-length DNA product (Fig. 2d) in high yield, demonstrating the compatibility of a UNA-modified DNA library in PCR amplification, which is a prerequisite for the subsequent cloning and sequencing steps involved in one-step selection of UNA-modified aptamers. Towards our goal of UNA-aptamer selection, based on our experience

on UNA nucleotides, we believe that the introduction of one UNA nucleotide is enough to impose significant conformational change and favour the formation of highly structured library members for enhanced target binding. This is supported by our earlier results on UNA-modified thrombin-binding aptamers where we saw increased binding relative to the unmodified parent aptamer after introduction of just one UNA nucleotide.^{8a}

In summary, we have demonstrated for the first time that certain DNA polymerases are able to copy limited stretches of a UNA-template and can incorporate up to two consecutive UNA-nucleotides in primer extension reactions. Terminator DNA polymerase was found to be the most efficient enzyme both for reading and incorporating UNA nucleotides. UNA-containing DNA template and library-based PCR was successfully accomplished using KOD DNA polymerase. As we have developed an efficient method for one-step selection of nucleic acid aptamers that relies on only one enzymatic protocol, we believe that our current finding of PCR amplification using a UNA-modified DNA library will allow us to identify highly flexible aptamers. Currently, we are focusing on one-step selection of UNA-modified DNA aptamers.

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