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COMMUNICATION

Synthesis of selenomethylene-locked nucleic acid (SeLNA)-modified oligonucleotides by polymerases†

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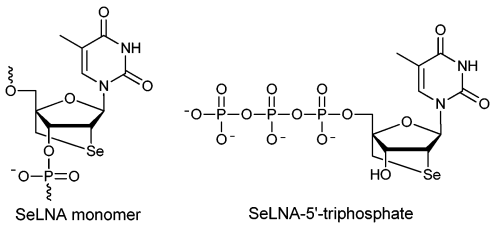
Enzymatic recognition of SeLNA nucleotides was investigated. KOD XL DNA polymerase was found to be an efficient enzyme in primer extension reactions. Polymerase chain reaction (PCR) amplification of SeLNA-modified DNA templates was also efficiently achieved by Phusion and KOD XL DNA polymerases.

Modified nucleotides have become a significant component in oligonucleotide-based therapeutic development as naturally occurring nucleic acids are especially vulnerable to nuclease attack which makes them incompatible as a therapeutic agent. Short single stranded DNA or RNA oligonucleotides called aptamers[1] are functional nucleic acids with significant potential in therapeutic, diagnostic and biosensor development. Aptamers display very high binding affinity and specificity to their targets, ranging from small molecules to complex proteins and even whole cells. Systematic Evolution of Ligands by EXponential enrichment (SELEX)[2] is a technique generally used to develop aptamers. Considering the recent developments in targeted nanotherapy and imaging technologies, the scope of aptamers with high bio-stability has increased tremendously. Aptamers composed of natural nucleotides are rapidly degraded by nucleases. Chemically-modified nucleotides are introduced into aptamers in order to overcome this limitation. However, as SELEX involves several enzymatic protocols, the applicability of the majority of the modified nucleotides is limited in the evolution of aptamers. A locked/bridged nucleic acid (LNA/BNA) nucleotide is one of the most prominent nucleotide analogues developed in recent years with remarkable properties.[3]

Since the first report of LNA/BNA,[4] a series of other derivatives have been reported by modifying the 2′-oxygen atom of the methylene bridge with nitrogen, sulphur or carbon to improve the binding affinity and nuclease resistance.[5] Selenium (Se) is an important atom introduced in the nucleic acids to facilitate the crystal growth and structure determination by X-ray crystallography, especially when it is placed at the 2′-position.[6] Using this strategy, several structures of DNAs, RNAs, and protein–nucleic acid complexes have been solved.[6a] We have recently prepared a new LNA/BNA analogue with a 'Se' atom at the 2′-position called SeLNA (Scheme 1).[7] We envision the development of SeLNA-modified aptamers with the scope to resolve the structure of an aptamer–target complex by X-ray crystallography and also to improve the nuclease resistance. Along this line, the enzymatic recognition of 2′-SeMe NTPs by RNA polymerases was recently reported.[8] Herein, we report the enzymatic recognition of SeLNA nucleotides by polymerases towards generating SeLNA-modified DNA aptamers.



Scheme 1 Structure of SeLNA nucleotide monomer and triphosphate.

To investigate the enzymatic recognition capabilities, a template containing six consecutive SeLNA-T nucleotides (T1, Fig. 1a) after the 19 nucleotide (nt) primer binding region was synthesized first to incorporate natural nucleotides opposite to SeLNA-T nucleotides. Five different polymerases,

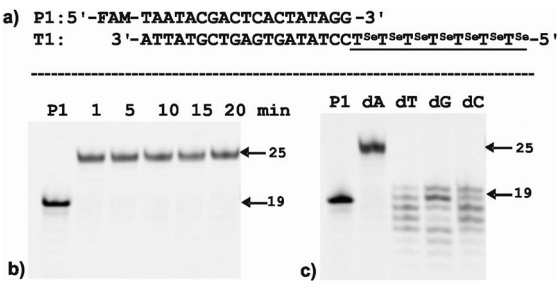


Fig. 1 Primer extension reactions using an SeLNA-template. (a) Primer and template sequences. (b) Reading of SeLNA-T template T1 to incorporate dA by KOD XL DNA polymerase. (c) Fidelity of reading SeLNA-T using all four natural nucleotides. SeLNA-T nucleotides are underlined and denoted by superscript 'Se'.

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KOD XL, Phusion High Fidelity (HF), Therminator, *Taq* and Klenow DNA polymerases, were tested by primer extension experiments. The extension product was analysed by 13% denaturing polyacrylamide gel electrophoresis for which the primer DNA was 5′-FAM-labeled. The experiment revealed that KOD XL DNA polymerase was the most efficient enzyme to read SeLNA-T nucleotides of the template strand and extend the primer to full-length (Fig. 1b). Other thermophilic B-family polymerases (Therminator and Phusion HF) tested also yielded the full-length product with slightly reduced yield (Fig. S1a and b, ESI†). Product degradation was observed in the case of Phusion HF DNA polymerase after five minutes of incubation. Out of the two family-A polymerases tested, Klenow DNA polymerase successfully extended the primer to full-length although the yield only improved upon prolonged incubation from one minute to fifteen minutes (Fig. S1c, ESI†). *Taq* DNA polymerase could only extend the primer DNA by three consecutive dA nucleotides failing to afford the full-length extension product even after fifteen minutes of incubation (Fig. S1d, ESI†).

To assess the polymerase fidelity in reading SeLNA-T and to incorporate the correct nucleotide (dA) by following strict Watson–Crick base pairing rules, extension experiments were conducted with KOD XL DNA polymerase using dTTP, dGTP and dCTP. While yielding the expected full-length product with dATP, the polymerase failed to extend the primer with the other three incorrect nucleotides after ten minutes of incubation (Fig. 1c). Prolonged incubation to twenty minutes only resulted in further degradation of the products (data not shown). These results suggest that the polymerase followed the necessary fidelity in reading SeLNA-T nucleotides. Experiments were also conducted by removing Mn^{2+} to investigate its importance in the reaction as Mn^{2+} is known to reduce the 2′-sugar discrimination.[9] The result showed that KOD DNA polymerase was able to yield the full-length extension product with good yield without $MnCl_2$ supplementation in the reaction (Fig. S2, ESI†).

Based on the initial results, we then performed extension experiments using a DNA template (T2, Fig. 2a) containing two SeLNA-T nucleotides along with other nucleotides as a

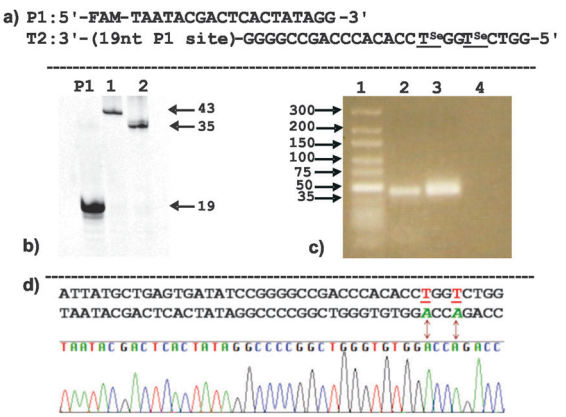

Fig. 2 Primer extension reactions using an SeLNA-template. (a) Primer and template sequences. (b) Extension by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs, lane 2: negative control without the 'T' nucleotide. (c) PCR amplification using the purified ssDNA. (d) Alignment of the sequencing chromatogram with the actual product sequence. SeLNA-T nucleotides are underlined and denoted by superscript 'Se'.

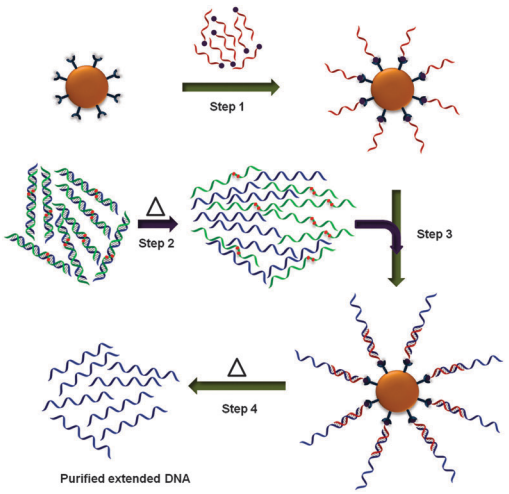

Fig. 3 Purification of the primer extended ssDNA. Step 1: biotinylated short probe DNA is immobilized on streptavidin coated magnetic beads; Step 2: phenol–chloroform extracted extension products were denatured; Step 3: the primer extended top strand is captured by the probe DNA; Step 4: after removing the undesired DNA, the complex is heated to 60 °C and the desired primer extended DNA strand is isolated.

first step towards the application of SeLNA-T nucleotides in aptamer selection by SELEX. KOD XL (Fig. 2b) successfully extended the primer to full-length. The accuracy of the extension product was further verified by sequencing. For sequencing the primer extension product, we isolated and purified the full-length primer extended single-stranded DNA strand (illustrated in Fig. 3) and PCR amplified (Fig. 2c), cloned and sequenced. The sequencing chromatogram clearly matched the actual sequences (Fig. 2d).

The first enzymatic step involved in aptamer selection by the conventional SELEX approach is to amplify the target bound aptamer candidate by PCR. Along this line, we performed PCR amplification using SeLNA-T-modified templates. Templates T2 with two SeLNA-T nucleotides (Fig. 4a) and T3, a 68 nt template with three SeLNA-T nucleotides (Fig. 4b), were used in this experiment. The result revealed that both the

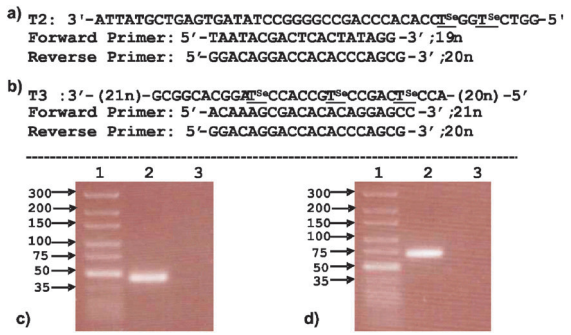

Fig. 4 PCR amplification using SeLNA-T modified DNA templates. (a) Primer sequences specific for template T2; (b) primer sequences specific for template T3; (c) agarose gel electrophoresis image of PCR using template T2, lane 1: DNA size markers in base-pairs, lane 2: PCR using T2, lane 3: PCR without using a template (negative control); (d) agarose gel electrophoresis image of PCR using template T3, lane 1: DNA size markers in base-pairs, lane 2: PCR using template T3, lane 3: PCR without using a template.

templates were successfully amplified using Phusion DNA polymerase (Fig. 4c and d). It is worth mentioning that PCR amplification using SeLNA-T triphosphate in place of dTTP in the reaction mixture with normal DNA templates or SeLNA-T-modified DNA templates was not successful.

Aptamer selection using modified nucleotide containing libraries requires converting the double-stranded DNA copies of the target binders after PCR to the corresponding ssDNA containing modified nucleotides. This is normally achieved by primer extension reaction using modified nucleotide triphosphate. To investigate the capability of polymerases to construct a DNA with SeLNA-T nucleotides, we synthesised the SeLNA-T nucleotide 5′-triphosphate (Scheme 1) using the protocol applied for the synthesis of LNA triphosphates[10] (see ESI† for full characterization). First, we performed the extension experiment using a template (T6, Fig. S5a, ESI†) designed to incorporate seven consecutive SeLNA-T nucleotides in the 3′-end of the primer. KOD XL, Therminator, Phusion, Klenow and *Taq* DNA polymerases were again tested for their capabilities to incorporate SeLNA-T nucleotides. The results showed that KOD XL and Therminator DNA polymerases were able to successively incorporate three and two SeLNA-T nucleotides, respectively (Fig. S3b and c, ESI†). Phusion and Klenow DNA polymerases were only able to incorporate one SeLNA-T nucleotide (Fig. S3d and e, ESI†) whereas *Taq* failed to accept the SeLNA-T nucleotide as a substrate. Later, an experiment was initiated to compare the polymerase recognition capabilities of SeLNA-T and normal LNA-T nucleotides. As expected, the results showed that KOD XL polymerase was able to incorporate three consecutive LNA nucleotides in very good yield and also to afford the full-length product in low yield whereas in the case of SeLNA nucleotides, the enzyme only extended the primer up to three nucleotides (Fig. S4, ESI†).

We further evaluated the efficacy of polymerase to incorporate SeLNA-T nucleotides along with other nucleotides using a longer DNA template. The designed 43 nt template (T4, Fig. 5a) has three sites of incorporation. Positive control (all four natural nucleotide triphosphates) and negative control (dATP, dCTP, dGTP) experiments were also performed in parallel to the SeLNA-T incorporation reactions. KOD XL DNA polymerase efficiently incorporated SeLNA-T nucleotides at the desired positions and extended the product to full-length (Fig. 5b). Prolonged incubation to 40 minutes produced the full-length extension product with very high yield although it was visible after 5 minutes. The extended primer strand with SeLNA-T nucleotide incorporation was isolated and purified as discussed earlier (Fig. 3) and amplified by PCR followed by cloning and sequencing. The sequencing chromatogram clearly matched the expected 'T' nucleotides at the desired positions (Fig. S5, ESI†). Another experiment was performed to investigate the multiple successive incorporation of SeLNA-T nucleotides along with other three nucleotide triphosphates. The designed template (T5, Fig. 5a) has eight consecutive sites of incorporation placed seven nucleotides away from the primer binding region. Remarkably, the results showed that KOD XL polymerase successfully incorporated eight consecutive SeLNA-T nucleotides and extended the primer to full-length (Fig. 5c). However, the yield was poor even after 45 minutes of incubation. Based on our extension experiments performed in the presence and absence of reducing agent DTT (no change observed, data not shown), it is worth mentioning that SeLNA nucleotides do not get oxidized during polymerase reactions.

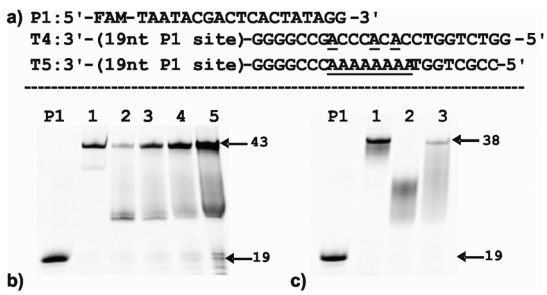

Fig. 5 Enzymatic incorporation using SeLNA-T-5′-triphosphate. (a) Primer and template sequences. (b) Extension by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs for 5 minutes, lanes 2–5: incorporation of SeLNA-T nucleotides (dATP, dGTP, dCTP and SeLNA–TTP in the mixture) at 5, 10, 20 and 45 minutes respectively. (c) Successive incorporation of SeLNA-T by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs for 5 minutes, lane 2: negative control without the 'T' nucleotide in the reaction mixture, lane 3: incorporation of SeLNA-T nucleotides (dATP, dGTP, dCTP and SeLNA–TTP in the mixture) after 45 minutes.

In summary, we have demonstrated that SeLNA-T nucleotides can be successfully accepted by polymerases. KOD XL DNA polymerase was found to be an efficient enzyme to construct oligonucleotide strands by reading SeLNA-T nucleotides of the template strand and also by incorporating SeLNA-T nucleotides using DNA templates. The findings reported here suggest that the evolution of SeLNA-T nucleotide-modified aptamers can be developed towards the elucidation of the aptamer structure by X-ray crystallography.

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