DNA Modification

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Synthesis and DNA-Polymerase Incorporation of Colored 4-Selenothymidine Triphosphate for Polymerase Recognition and DNA Visualization**

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DNA polymerase replicates cellular DNA in a template-dependent manner by using deoxyribonucleotide triphosphates (dNTPs). Specific recognition occurs through base pairing and size-and-shape matching.[1] Extensive studies have focused on the recognition and stability of the DNA double-stranded structure and on the interaction between the dNTP substrate, the template, and DNA polymerase.[2] In the chemogenetic investigation of the structure and function of nucleic acids, we pioneered the atom-specific substitution of an oxygen atom in the nucleic acid (atomic radius: 0.73 Å) with selenium (1.16 Å), which is also in Group 16 of the periodic table, as an atomic probe.[3] Our recent replacement of the oxygen atom at the 4-position of thymidine with a selenium atom revealed that the DNA duplex is flexible enough to accommodate the large selenium atom and that the C=Se functionality is relatively stable.[4] Furthermore, we discovered that the 4-Se atom forms a selenium-mediated hydrogen bond with the 6-amino group of adenosine (Se···H–N), and that the crystal structure of the DNA molecule with the Se-modified nucleobase is virtually identical to that of the corresponding native DNA.[4]

We have now discovered colored 4-Se-thymidine 5′-triphosphate (^{Se}TTP; Figure 1). We formed yellow ^{Se}TTP from colorless native TTP by altering a single atom and observed the recognition and incorporation of ^{Se}TTP by DNA polymerase. The visualization of DNA is of interest and importance for many biochemical, biological, and medical applications,[5–7] such as gene-expression analysis and the detection of human diseases and pathogens. DNA is usually visualized by using intercalated dye molecules[8,9] and specific DNA probes tagged with fluorescent moieties.[5,10,11] However, these often bulky intercalated dye molecules and tethered fluorescent moieties can cause the perturbation of DNA duplex structures. We describe herein the synthesis of colored ^{Se}TTP and its efficient incorporation into DNA molecules by DNA polymerase. This method should lead to the development of novel DNA probes for visualization without structure perturbation.

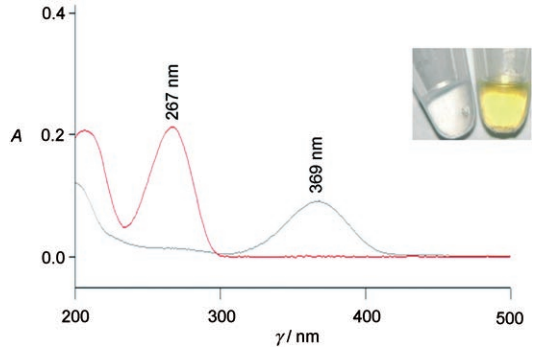
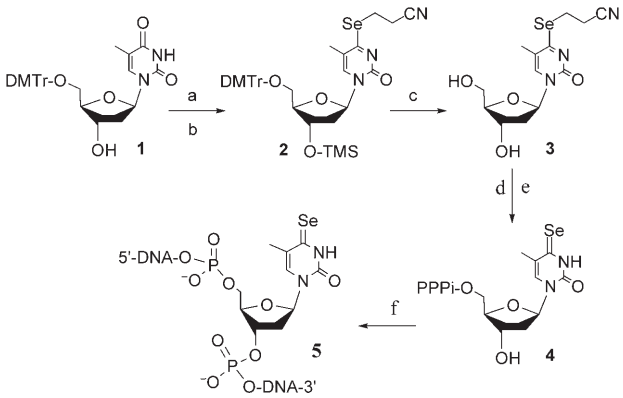


Figure 1. UV spectra of TTP (red, λ_{max} = 267 nm) and ^{Se}TTP (gray, λ_{max} = 369 nm). Inset: left: TTP (colorless); right: ^{Se}TTP (yellow).

Our previous attempts to synthesize ^{Se}TTP failed as a result of deselenization. Following the development of a useful protecting group for Se,[12] we finally completed the synthesis of ^{Se}TTP (Scheme 1). This innovative strategy enables both the incorporation of Se and the protection of **2**. After the removal of the 5′-DMTr and 3′-TMS protecting groups, the 5′-OH group of **3** was phosphorylated by treatment with $POCl_3$[13] followed by pyrophosphate, and the cyanoethyl protecting group was removed under mild conditions by treatment with K_2CO_3. The cyanoethyl group may also be removed prior to the phosphorylation step.



Scheme 1. Chemical synthesis of ^{Se}TTP (**4**) and enzymatic synthesis of ^{Se}T-containing DNA (**5**): a) *N*-trimethylsilylimidazole; 1,2,4-triazole, $POCl_3$, Et_3N; b) $(NCCH_2CH_2Se)_2$, $NaBH_4$, EtOH; c) 80% acetic acid; d) $POCl_3$, Me_3PO_4; tri-*n*-butylamine, pyrophosphate, *N*,*N*-dimethylformamide; H_2O; e) aqueous K_2CO_3 (0.05 M); f) template-dependent DNA polymerization. DMTr = 4,4′-dimethoxytrityl, TMS = trimethylsilyl.

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The Se-modified triphosphate was purified by HPLC, and its identity was confirmed by HPLC and HRMS (see the Supporting Information). We found that the selenium functionality of ^{Se}TTP is stable in air under aqueous conditions. The UV spectrum of ^{Se}TTP ($\lambda_{max} = 369$ nm, $\varepsilon = 1.8 \times 10^4\,M^{-1}cm^{-1}$, yellow) reveals a larger absorption and a red shift of 100 nm relative to that of TTP ($\lambda_{max} = 267$ nm, $\varepsilon = 9.2 \times 10^3\,M^{-1}cm^{-1}$, colorless). A smaller red shift was observed for the 4-S-thymidine nucleotide ($\lambda_{max} = 335$ nm, $\varepsilon = 2.0 \times 10^4\,M^{-1}cm^{-1}$, colorless)[14,15] relative to the absorption of the thymidine nucleotide. The color of the Se-modified nucleotide is probably due to the ease of delocalization of the selenium electrons on the nucleobase. Thus, less energy is required for electron excitation, and the large red shift in the UV spectrum results.

The Se-triphosphate is a good substrate for DNA polymerases. It is recognized efficiently by the high-fidelity Klenow fragment of *E. coli* DNA polymerase I (Figures 2 and 3).[16] We incorporated the Se-modified triphosphate into DNA molecules on several different DNA templates and found that the polymerized Se-DNA is yellow. By using a short DNA template (T1) we incorporated ^{Se}TTP into a short DNA primer (P1; Figure 2A) and confirmed the incorporation of the Se-modified nucleotide by MS analysis (Figure 2B). The mass difference between the DNA molecules extended with a single ^{Se}TTP or TTP unit is 63 Dalton, which indicates the incorporation of the Se-modified thymidine base (mass difference for the replacement of O with Se: 79−16 = 63 Dalton).

With a longer DNA template, we examined the incorporation of TTP and ^{Se}TTP into DNA with time (Figure 3A) and found that the ^{Se}TTP polymerization efficiency is similar to that of native TTP (Figure 3B). Both the Klenow fragment and the exo-Klenow fragment incorporate ^{Se}TTP with high efficiency; no significant difference between these two enzymes was observed. Thus, the high-fidelity enzymes[16] are capable of recognizing the Se-modified nucleobase. This result is consistent with the incorporation of S-modified thymidine by a polymerase.[2b,c]

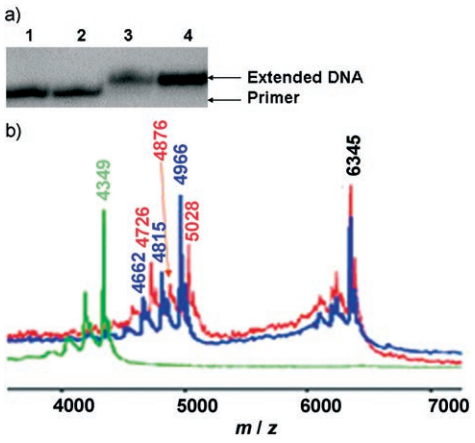

Figure 2. Incorporation of ^{Se}TTP or TTP by the exo-Klenow enzyme on a DNA template (T1, 3′-ATCGCCCAACGACCACCTTGG-5) with a primer (P1, 5′-^{32}P-TAGCGGGTTGCTGG-3′). a) All lanes contain P1 and T1. Lane 1: no enzyme; lane 2: no TTP; lane 3: ^{Se}TTP and enzyme; lane 4: TTP and enzyme. b) MS spectra of P1 (green; *m/z* calc. for P1: 4349.8 $[M-H]^-$; found: 4349), the T-extended DNA (O-15-mer, blue; *m/z* 4662, 4815, 4966), and the ^{Se}T-extended DNA (Se-15-mer, red; *m/z* 4726, 4876, 5028). T1: *m/z* 6345 (peak labeled in black). Mass differences between the Se-15-mer and the O-15-mer: 4726−4662 = 64, 4876−4815 = 61, 5028−4966 = 62 (see the Supporting Information).

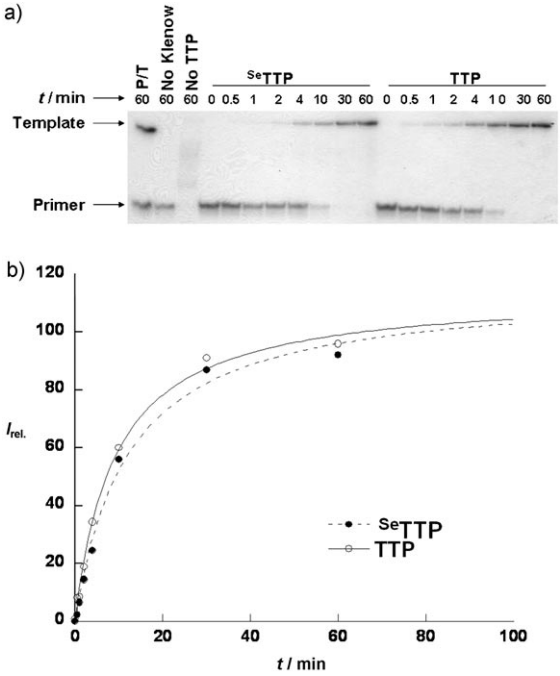

Figure 3. Time course of the incorporation of TTP and ^{Se}TTP into DNA with a DNA primer (P2, 5′-GCGTAATACGACTCACTATAG-3′) and the Klenow enzyme on a DNA template (T2, 3′-CGCATTATGCTGAGTGATATCCG-TTGGACTACTCCGGCTTTCCGGCTTTGCATGT-5′). a) Gel electrophoresis; b) plot of the incorporation of TTP and ^{Se}TTP into DNA with respect to time.

In summary, we have synthesized a visible (yellow) nucleoside triphosphate, 4-Se-thymidine 5′-triphosphate, by changing a single atom in the parent base. DNA polymerase recognizes efficiently this Se-modified triphosphate and the Se-mediated hydrogen bond (Se···H−N). The incorporation of ^{Se}TTP into DNA yields colored DNA and occurs with the same level of efficiency as the incorporation of natural TTP. The spectroscopic properties of visible ^{Se}TTP and its polymerization into DNA will shed new light on specific recognition governed by the size and shape of bases, base pairing, and stacking interactions, and on the efficiency and fidelity of DNA replication. Moreover, the Se-DNA and its visualization have great potential in the determination of nucleic acid crystal structures via multiwavelength anomalous dispersion (MAD) phasing,[3e,4,17] as well as for the nucleic acid based detection of human diseases and pathogens.

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