論文内容の要旨

Enzymatic Synthesis of Artificial Metallo-DNAs Utilizing Template-independent DNA Polymerases

(鋳型非依存性 DNA ポリメラーゼを活用した金属錯体型人工 DNA の酵素合成)

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1. Introduction

DNA molecules self-assemble precisely to form two or three-dimensional structures (e.g. DNA origami, DNA polyhedra) through complementary hydrogen bonding. Consequently, DNA has gained a great interest as one of the promising materials in terms of nanotechnology. Incorporation of metal complex into DNA structures allows for the construction of high-ordered nanostructures that show higher thermal stability. Such artificial metallo-DNAs possess ligand-bearing artificial nucleobases (e.g. hydroxypyridone, **H**), which form metal-mediated base pairs (e.g. **H**–Cu^{II}–**H**) in the presence of metal ions (Fig. 1). Due to their unique geometry, dynamics and high thermal stability, metallo-DNAs have recently attracted broad interest as scaffolds to build DNA-based molecular nanomachines and devices.

In this study, I have developed a novel synthetic method of metallo-DNAs using DNA polymerases (Fig. 1). Compared with conventional chemical synthetic methods, enzymatic synthetic methods have characteristics that the reaction proceeds with high efficiency and enzymes undergo post-synthetic, site-selective modification of DNA hybridized structures. Here, among various kinds of polymerases, template-independent DNA polymerases (terminal deoxynucleotidyl transferase, TdT) were chosen. In high contrast to the other polymerases, TdT polymerases can accept unnatural substrates to selectively elongate single-stranded DNAs and thus have been extensively used to incorporate functional building blocks into desired positions of DNA architectures.

To synthesize artificial metallo-DNAs by utilizing TdT polymerase, a hydroxypyridone ligand-bearing artificial nucleotide triphosphates (d**H**TP) was synthesized as a substrate. Subsequently, it was investigated that TdT polymerized d**H**TPs to provide ligand-bearing artificial DNAs and the artificial DNAs formed metallo-DNAs in the presence of Cu^{II} ions. Furthermore, in order to validate the usability of the enzymatic synthesis, post-synthetic and site-selective modification of DNA duplexes was demonstrated.

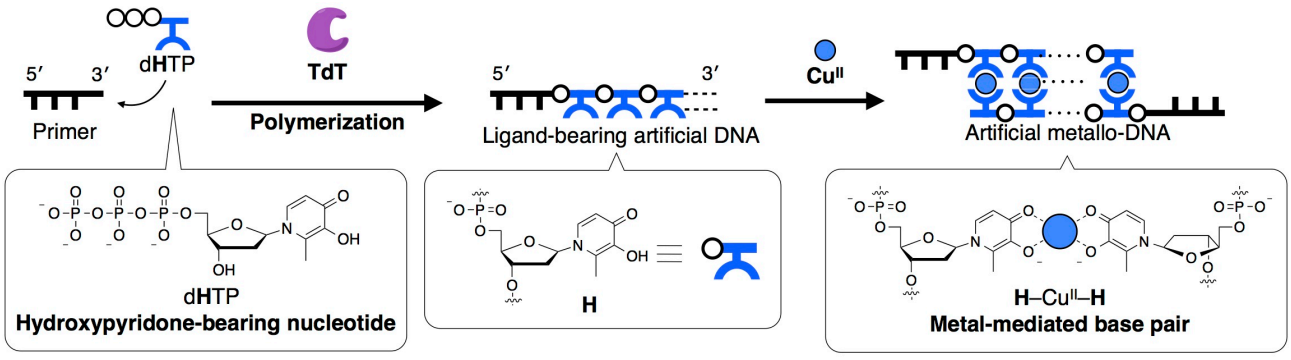
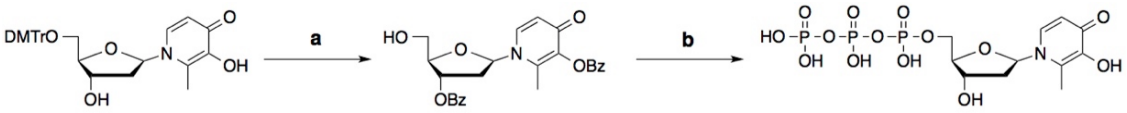


Fig. 1 Schematic representation for enzymatic synthesis of artificial metallo-DNAs.

2. Synthesis of an Enzymatic Substrate (dHTP)

As a substrate for the TdT, the triphosphate derivative of the **H** nucleotide (d**H**TP) was prepared by the conventional Eckstein method for selective 5′-phosphorylation (Scheme 1). DMTr-protected nucleoside **1** was prepared according to the previous report and then benzoylated and detritylated to provide a protected nucleoside **2**. Subsequently, the bis-benzoylated nucleoside **2** was selectively phosphorylated at the 5′-position to yield the desired triphosphate d**H**TP as a triethylammonium salt. Although by-products were generated including a nucleotide monophosphate, they were successfully separated from a desired triphosphate d**H**TP by the anion-exchange column chromatography. Subsequent reversed-phase HPLC purification afforded d**H**TP that was pure enough for the enzymatic reactions, as characterized by NMR and mass spectrometry (5.3%, 4 steps from **2**).

Scheme 1 Synthesis of the hydroxypyridone-bearing nucleotide triphosphate (d**H**TP).



(a) (i) Benzoic anhydride, DMAP, dry pyridine, room temperature; (ii) 2.2% CCl_3COOH/CH_2Cl_2, 0 °C; (b) (i) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, dioxane, pyridine, room temperature; (ii) bis(tri-*n*-butylammonium)pyrophosphate, *n*-tributylamine in DMF; (iii) 1% I_2 in pyridine/H_2O; (iv) 1 M NaOH; (v) 0.1 M TEAA buffer (pH 7.0).

3. Enzymatic Synthesis of Ligand-bearing Artificial DNAs

Synthesis of ligand-bearing artificial DNAs was investigated with a 6-carboxyfluorescein (FAM) labeled DNA primer (FAM-dT_{20}) in the presence of template-independent DNA polymerase (TdT). The primer (5 μM) and **H** nucleotide triphosphate (d**H**TP, 100 μM) were incubated with TdT enzyme (2 U/μM) at 37 °C for 24 h. The resulting products were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 2). The observed ladder pattern showed evidence that TdT enzyme catalyzed the sequential polymerization of d**H**TP, which provided the artificial DNAs tailed with 3–7 **H** nucleotides (5′-FAM-dT_{20}-**H**$_n$-3′ (n = 3–7)). This result was consistent with the result of MALDI-TOF mass spectmetiric analysis that showed a series of signals with intervals of m/z = 303 which was ascribed as an **H** nucleotide monophosphate ($C_{12}H_{14}NO_7P$).

One of the main products possessing five **H** nucleotides (5′-FAM-dT_{20}-**H**$_5$-3′, **ODN-1**) was isolated and characterized by MALDI-TOF mass spectrometry. The detected m/z value was 8077.5, which was consistent with the calculated value 8075.4. UV absorption spectral analysis was further conducted as shown in Fig. 3A. The difference spectrum (red line) between **ODN-1** and the primer had an absorption maximum at 282 nm, which was consistent with the spectrum of fivefold **H** nucleoside monomers (blue line, λ_{max} = 282 nm). This result confirmed that the **H** nucleotides were polymerized with their ligand moiety intact. Thus, it was concluded that the ligand-bearing artificial DNAs possessing **H** nucleotides were successfully synthesized by TdT polymerase.

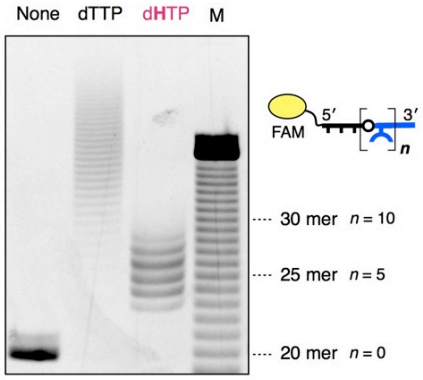


Fig. 2 Characterization of the reaction products obtained by the TdT-aided extension of a FAM-labeled primer strand using d**H**TP. Denaturing polyacrylamide gel electrophoresis (PAGE) analysis. The bands were detected by FAM fluorescence. [FAM-dT_{20}] = 5.0 μM (primer), [d**H**TP] = 100 μM, [TdT] = 2 U/μL in 20 mM Tris-acetate buffer (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KOAc, 37 °C, 24 h.

Subsequently, in order to prove metal complexation ability of **ODN-1**, complexation of **ODN-1** with Cu^{II} ions was elucidated by UV absorption spectral analysis. **ODN-1** was combined with Cu^{II} ions (i.e. 0.5 equiv. per **H** nucleotide) in a neutral buffer. As shown in Fig. 3B, a difference spectrum (red line) between the complexation product and the primer was well fitted with a spectrum of the **H**–Cu^{II}–**H** complex (blue line, λ_{max} = 303 nm). This result confirmed that artificial metallo-DNA duplexes, which have metal-mediated base pairs, was successfully constructed with the enzymatically-synthesized DNA strands in the same manner as conventional chemical DNA synthesis.

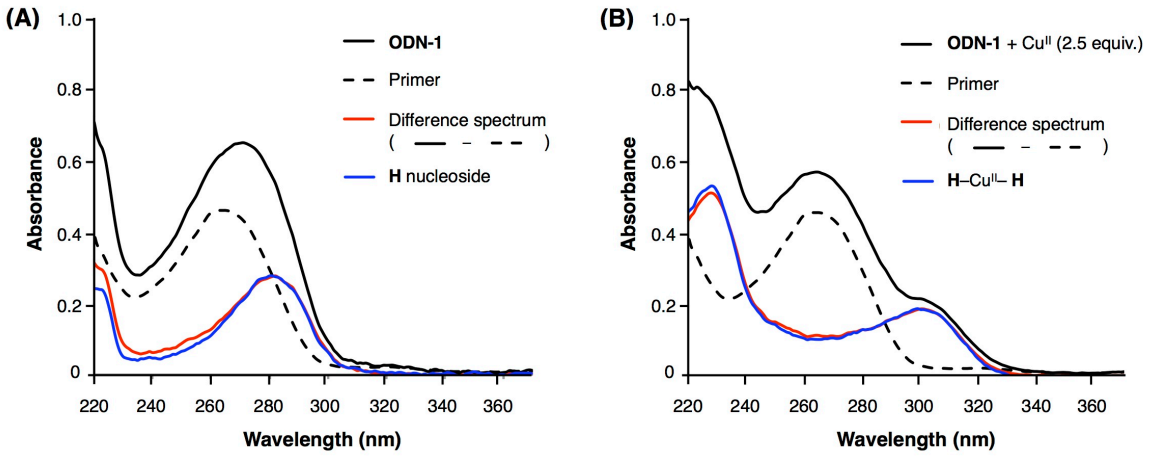


Fig. 3 UV absorption spectra of the isolated product, 5′-FAM-dT_{20}-$\mathbf{H}_5$-3′ (**ODN-1**) (36 μM) in the absence **(A)** and presence **(B)** of Cu^{II} ions (90 μM, 2.5 equiv.) (black solid lines). A spectrum of the primer (36 μM) (black broken lines), difference spectra (red lines), a spectrum of **H** nucleoside (180 μM) (A, blue line) and that of **H**–Cu^{II}–**H** complex (90 μM) (B, blue line) are overlaid. In 100 mM HEPES buffer (pH 7.0), 500 mM NaCl, l = 0.1 cm, at room temperature. Concentrations of the DNAs were determined based on the absorbance of the FAM moiety at λ = 495 nm.

It was revealed that the enzymatic DNA synthesis stalled when about five **H** nucleotides were incorporated by TdT enzyme. Actually, TdT incorporated neither dTTP nor d**H**TP when **H**-tailed **ODN-1** was used as the primer. This result implied that artificial DNAs possessing several **H** nucleotides have a low affinity to the enzyme and thus did not act as a primer for further elongation. It should be noted that the TdT-catalyzed reaction further proceeded in the presence of the high concentration of DNAs to yield DNA oligomers tailed with about 10 **H** nucleotides. It is most likely that increasing the concentration forced the DNA to bind the enzyme and consequently facilitated the reaction progress. Thus, the limitation in the length of the product was overcome and so the synthesis of much longer ligand-bearing artificial DNAs would be realized by the synthetic method developed here.

4. Post-synthetic modification of the DNA duplexes by TdT and metal-mediated assembly of the modified duplexes

One of the practical advantages of the enzymatic reaction is to allow for post-synthetic modification of DNA nanostructures. Consequently, the post-synthetic incorporation of artificial ligand-bearing nucleotides by TdT enzyme would be a powerful tool to construct DNA-based materials with metal-mediated base pairs. Herein, post-synthetic modification of simple DNA duplexes was investigated as a model for DNA-based nanoarchitectures (Fig. 4A).

In the presence of d**H**TP and TdT enzyme, DNA duplex (**ODN-2**·**ODN-3**) with 3′-protruding end was tailed with about five **H** nucleotides in the same manner as single-stranded DNA primer. In contrast, the reaction of 3′-blunt DNA duplex did not proceeded at all. Accordingly, it is expected that the enzymatic DNA synthesis developed here will be usable for site-selective modification of 3′-protruding end of DNA nanostructures.

Subsequently, the metal complexation behavior of **H**-modified duplex was elucidated (Fig. 4A). The modified DNA duplex possessing five **H** nucleotides on average were mixed with Cu^{II} ions (i.e. 0.5 equiv. per **H** nucleotide). The band on the gel image shifted to the position of a higher molecular weight (Fig. 4B, lane 3), which approximately corresponded to twofold molecular weight of the metal-free monomeric duplex. This result indicated that a dimeric complexation structure was constructed by formation of artificial metallo-DNA duplex through metal-mediated **H**–Cu^{II}–**H** base pairing. The formation of metallo-DNA duplexes was also confirmed by UV absorption spectroscopy. Furthermore, Cu^{II} titration experiments with PAGE analysis showed that the dimeric structure was formed quantitatively and this structure was stable in the presence of excess amounts of Cu^{II} (i.e. 1.0 equiv. per **H** nucleotide). Subsequent addition of EDTA to remove Cu^{II} ions regenerated the monomeric duplex (lane 4). These results confirmed that the enzymatically modified duplexes can assemble and disassemble in response to addition and removal of Cu^{II}.

Taken together, it is expected that the modification method established here will be further applied for the metal-responsive assembly of DNA-based nanomaterials such as DNA origami architectures, DNA-modified proteins and DNA-coated nanoparticles.

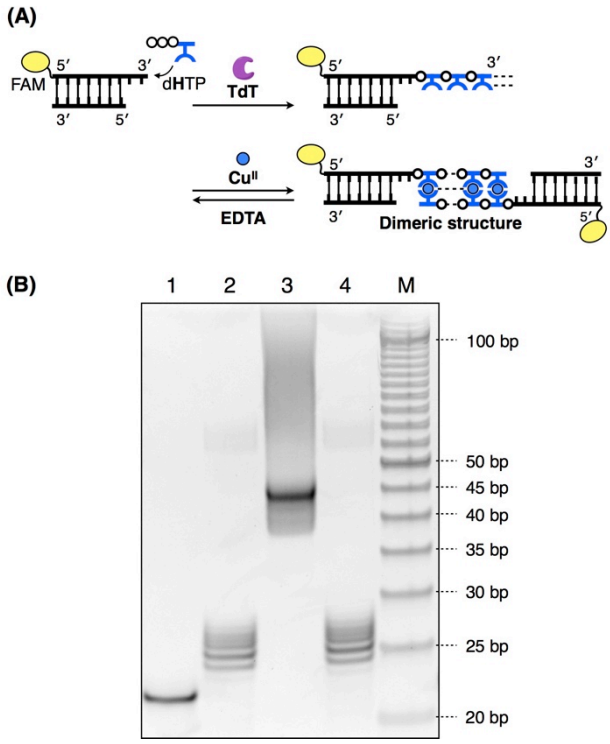


Fig. 4 (A) Schematic illustration of enzymatic modification of a DNA duplex and metal-mediated assembly of the duplexes. **(B)** Native PAGE analysis of the resulting structures. (Lane 1) the starting duplex (**ODN-2**·**ODN-3**) (32 μM), (lane 2) after the enzymatic reaction, (lane 3) after addition of Cu^{II} ions (90 μM), (lane 4) after subsequent addition of EDTA (1 mM), (M) double-stranded DNA markers. The bands were detected by FAM fluorescence. **ODN-2**: FAM-5′-GAA GGA ACG TAC ACT CGC AGT T-3′ (22 mer), **ODN-3**: 5′-CTG CGA GTG TAC GTT CCT TC-3′ (20 mer). The condition of the enzymatic reaction was the same as that for Fig. 2.

4. Conclusion

In this study, a hydroxypyridone ligand-bearing artificial nucleotide triphosphate (d**H**TP) was synthesized as a substrate of TdT polymerase. By chemical and bio-related analytical methods, it was revealed that TdT are useful to sequentially polymerize d**H**TP for synthesis of ligand-bearing artificial DNAs. It was found that ligand moieties of the artificial DNA remained intact. The enzymatically-synthesized DNAs formed **H**–Cu^{II}–**H** base pair quantitatively to construct the metallo-DNAs as was the case of chemically synthesized DNA. In addition, the enzymatic DNA synthesis was stalled when about five **H** nucleotides were polymerized, probably because the elongated artificial DNA had a low affinity with the enzyme. It was revealed that further polymerization reaction was facilitated with the high concentration of DNAs by forcing DNAs to bind to the enzyme. Furthermore, in order to reveal the usability of the enzymatic synthesis, post-synthetic and site-selective modification of DNA duplexes was demonstrated. The reaction proceeded only from the 3′-protruding end. Therefore, this strategy can be applied to site-selective modification for the 3′-protruding end on the DNA-based materials. The modified DNA duplex self-assembled in response to formation of complexes with Cu^{II} ions. Consequently, TdT-catalyzed post-synthetic modification would be readily applied for DNA-modified nanomaterials and biomacromolecules. The method established here will allow for the development of DNA-based materials whose structures and functions can be regulated in response to metal coordination.