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We have developed a novel method to synthesise artificial ligand-bearing DNAs utilising a template-independent DNA polymerase. Hydroxypyridone ligand-bearing nucleotides (H) were successively appended to DNA primers by the enzyme. The resulting strands, tailed with H nucleotides, formed Cu^{II}-mediated metallo-DNA duplexes through the formation of metal-mediated artificial base pairs (H–Cu^{II}–H).

DNA molecules hybridise in a sequence specific manner through hydrogen-bonded base pairing. This remarkable feature allows for programmable self-assembly of DNA strands, which has made a major contribution to the construction of DNA-based nanoarchitectures and nanodevices.[1] We and others have developed noncanonical metal-mediated base-pairing systems, which can embed further functionalities to DNA materials.[2] A metallo-base pair consists of two ligand-type nucleobases and a central metal ion, crosslinking two strands through metal coordination bonding. For instance, a natural thymine (T) base is known to form an Hg^{II}-mediated base pair (T–Hg^{II}–T) through a selective binding of an Hg^{II} ion to the N3 atoms.[3]

Metal-mediated base pairs comprised of unnatural ligand-bearing artificial nucleobases have gained much interest in the past fifteen years.[2] Dozens of artificial ligand-bearing nucleosides have been developed so far and incorporated into DNA strands by conventional chemical synthesis. These artificial nucleosides form metal-mediated base pairs with various metal ions including Cu^{II}, Ag^{I} and Gd^{III}.[2,4] Such metallo-base pairs have substantial advantages as building blocks of DNA nanoarchitectures because they are fully orthogonal to the natural base-paring systems. A leading example is a Cu^{II}-mediated hydroxypyridone base pair (**H**–Cu^{II}–**H**), formed through 2:1 complexation of hydroxypyridone-bearing nucleosides (**H**) with a square-planar Cu^{II} ion (Fig. 1).[5] The **H**–Cu^{II}–**H** base-pairing provides the Cu^{II}-dependent duplex stabilisation[5] and can be applied for DNA-templated one-dimensional metal assembly[6] as well as for the regulation of the electroconductivity of DNA devices.[7]

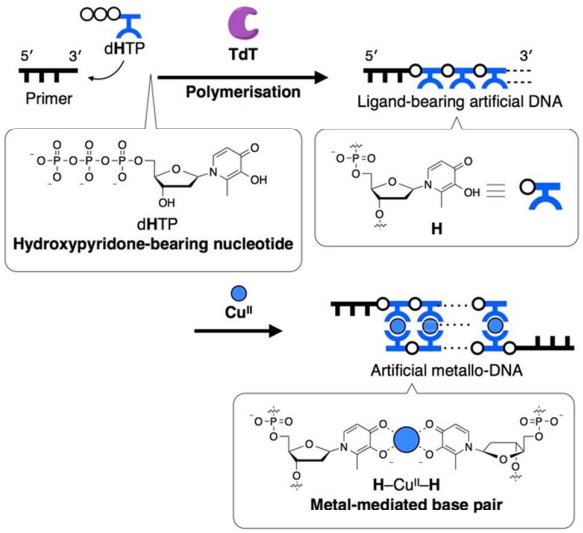

Fig. 1 Schematic illustration of enzymatic synthesis of ligand-bearing artificial DNAs that form Cu^{II}-mediated hydroxypyridone base pairs (**H**–Cu^{II}–**H**). TdT: terminal deoxynucleotidyl transferase.

In this study, we have developed a novel method to synthesise ligand-bearing artificial DNAs via an enzymatic DNA polymerisation reaction. Enzymatic DNA synthesis is one of the fundamental and powerful tools not only in molecular biology but also in the development of DNA-based materials.[8] Enzymatic incorporation of ligand-bearing nucleotides through metal-mediated base pairing has been recently demonstrated using template-dependent DNA polymerases.[9] Herein, we utilised a template-independent polymerase, terminal deoxynucleotidyl transferase (TdT),[10] to synthesise ligand-bearing DNA strands, which subsequently form metallo-DNA duplexes through metal-mediated base pairing (Fig. 1). TdT originally catalyses the sequential addition of natural nucleotide triphosphates at the 3′-terminus of single-stranded primers without template DNA strands. However, this enzyme is also known to accept modified nucleobases[11–14] and even unnatural ones.[15,16] Consequently, TdT has been extensively applied for the 3′-modification of oligonucleotides with fluorophores[12,15] and redox-active functionalities[13] as well as for the 3′-capping to confer exonuclease resistance.[17] We then examined the TdT-aided

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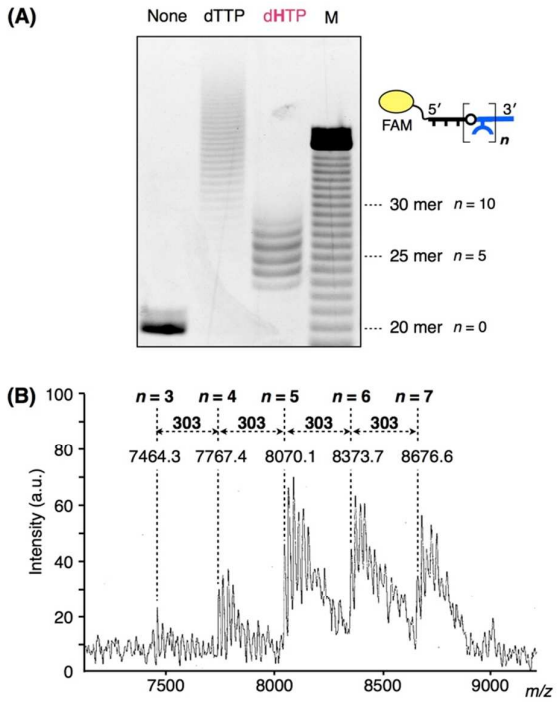

Fig. 2 Characterisation of the reaction products obtained by the TdT-aided extension of a FAM-labelled primer strand using d**H**TP. **(A)** Denaturing polyacrylamide gel electrophoresis (PAGE) analysis. The bands were detected by FAM fluorescence. **(B)** MALDI-TOF mass spectrometric analysis. Sodium adducts were also detected. [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.

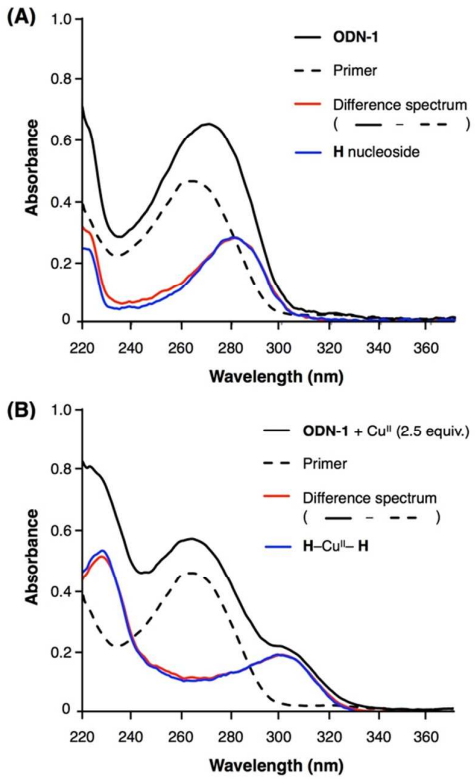

Fig. 3 UV absorption spectra of the isolated product, 5′-FAM-dT_{20}-**H**$_5$-3′ (**ODN-1**) (36 μM) in the absence **(A)** and presence **(B)** of Cu^{II} ions (90 μM, 2.5 equiv.) (black solid lines). A spectrum of the primer (36 μM) (black broken lines), difference spectra (red lines), a spectrum 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.

extension of a primer using hydroxypyridione-bearing nucleotides (**H**) to synthesise ligand-bearing artificial DNA strands.

As a substrate for the TdT, the triphosphate derivative of the **H** nucleoside (d**H**TP) was prepared by the conventional Eckstein method[18] (Scheme S1, ESI†). Since the **H** nucleobase is similar to the natural thymine base in size and shape, TdT was expected to incorporate d**H**TP to efficiently provide ligand-bearing DNA oligomers, which can be subsequently subjected to metal complexation with Cu^{II} ions to form metallo-DNA duplexes.

TdT-catalysed incorporation of d**H**TP into DNA strands was examined with a FAM-labelled primer strand (FAM-dT_{20}). The primer (5 μM) and 20 equiv. of the triphosphate (100 μM) were incubated with TdT (2 U/μM) at 37 °C for 24 h. The reaction products were analysed by denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 2A). A characteristic ladder pattern was observed on the gel, which indicated the generation of extended DNA oligomers. Each band was ascribable to the oligonucleotides tailing with 3–7 **H** nucleotides, namely 5′-FAM-dT_{20}-**H**$_n$-3′ (n = 3–7). The average extension length (n) was roughly estimated to be ca. 5 bases from the band intensities. This result suggested that TdT recognised d**H**TP as a substrate and successfully catalysed the polymerisation reaction.

The incorporation of d**H**TP into the strands was significantly slower than those of the natural nucleotide triphosphates (Figs. 2A and S1, ESI†). Increasing reaction time did not result in further extension of the **H** oligomer despite the existence of a substantial amount of d**H**TP (Fig. S2A, ESI†). Moreover, the increase in d**H**TP concentration little affected the extension length of the products (Fig. S2B, ESI†). Taken all together, it appears that the TdT-catalysed reaction was halted after several **H** nucleotides were appended.[19] Considering that TdT requires at least three nucleotides as a primer,[20] it is likely that the extended oligonucleotides, containing ~5 unnatural **H** nucleotides at the 3′ ends, were no longer recognised by the enzyme.[21]

The reaction products were then characterised by MALDI-TOF mass spectrometry (Fig. 2B). The mass spectrum of the reaction mixture showed a series of signals with intervals of m/z = 303 which corresponds to the molecular weight of a hydroxypyridone-bearing nucleotide monophosphate ($C_{12}H_{14}NO_7P$). The detected m/z values were consistent with the calculated values for the oligonucleotides possessing 3–7 **H** nucleotides. Consequently, the mass spectrometric analysis established the sequential addition of 3–7 d**H**TPs by the enzyme.

One of the main products which possesses five **H** nucleotides (5′-FAM-dT_{20}-**H**$_5$-3′, **ODN-1**) was isolated from the gel and further characterised by UV absorption spectroscopy (Fig. 3A). The absorbance of **ODN-1** (black solid line) was significantly larger than that of the primer (black broken line). The difference spectrum (red line) had an absorption maximum at 282 nm and was well fitted with the spectrum of fivefold **H** nucleoside monomers (blue line, λ_{max} = 282 nm). This result confirmed that the **H** nucleotides were condensed with their ligand moiety intact. Thus, the ligand-bearing artificial DNA oligomers were successfully synthesised by the enzyme.

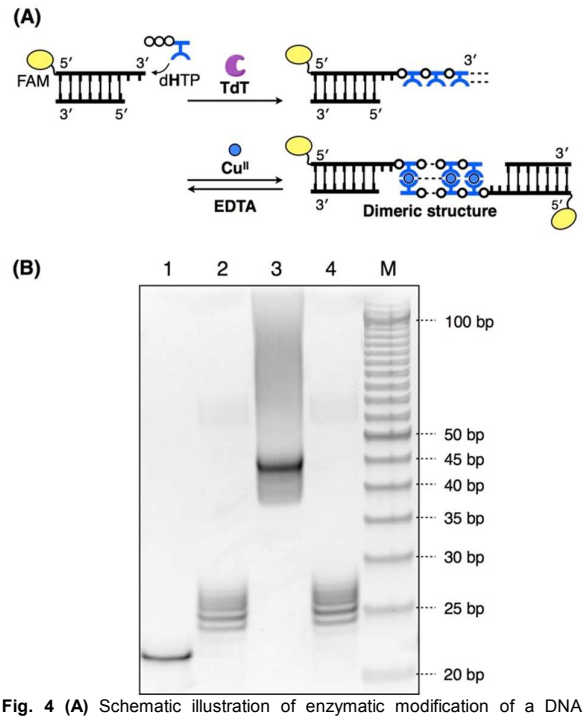

Fig. 4 (A) Schematic illustration of enzymatic modification of a DNA duplex and metal-mediated assembly of the duplexes. **(B)** Native PAGE analysis of the resulting structures. (Lane 1) the starting duplex (**ODN-2**·**ODN-3**), (lane 2) after the enzymatic reaction, (lane 3) after addition of 2.8 equiv. of Cu^{II} ions, (lane 4) after subsequent addition of EDTA, (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.

Subsequently, metal complexation behaviour of the enzymatically synthesised DNA was elucidated by UV spectroscopic analysis. **ODN-1** was combined with 2.5 equiv. of Cu^{II} ions (i.e. 0.5 equiv. per **H** nucleotide) in a pH 7.0 buffer. As shown in Fig. 3B, a new absorption band appeared around 303 nm (black solid line), which is indicative of the formation of an **H**–Cu^{II}–**H** base pair (i.e. a 2:1 complex of **H** nucleobase and Cu^{II}).[5] A difference spectrum (red line) of the complexation product and the primer strand was well fitted with a spectrum of 2.5 equiv. of the **H**–Cu^{II}–**H** complex (blue line). This result suggested that the metal-mediated **H**–Cu^{II}–**H** base pairs were quantitatively formed to provide a metallo-DNA duplex structure as was the case with chemically synthesised **H**-oligomers.[6a]

One of the practical advantages of the enzymatic DNA synthesis is the ability to allow post-synthetic modification of DNA-based materials.[8] TdT-catalysed DNA extension has recently been applied for the modification of DNA block copolymer nanoparticles[22] and DNA origami nanostructures.[23] Thus, the post-synthetic incorporation of artificial ligand-bearing nucleotides by TdT would be a powerful tool to endow DNA hybrid materials with metal-responsive properties. The usability of the enzymatic synthesis demonstrated above was verified using a simple DNA duplex consisting of a 22-mer primer (**ODN-2**) and a complementary 20-mer strand (**ODN-3**). The duplex has a 3'-protruding end, from which the primer extension is expected to start (Fig. 4A). After incubation with 20 equiv. of d**H**TP for 24 h, the reaction products were analysed by native PAGE (Fig. 4B). As a result, d**H**TP was efficiently appended to the duplex (lane 2) similarly to the case with a single-stranded primer. In contrast, the enzymatic reaction with a blunt-end duplex did not provide any extended product (Fig. S11, ESI†). This suggests that only the 3'-protruding end was tailed with **H** nucleotides. The number of the tailed **H** nucleotides was estimated to be ca. 5.5 in average based on its UV absorption spectrum (Fig. S7, ESI†).

With the **H**-modified duplex in hand, we then examined metal-mediated assembly of the DNA duplex. When 2.8 equiv. of Cu^{II} ions were added (i.e. 0.5 equiv. per **H** nucleotide), the band on the gel image shifted to a higher molecular weight (Fig. 4, lane 3), which approximately corresponds to twofold molecular weight of the metal-free duplex. This indicates that a dimeric structure was formed through metal-mediated **H**–Cu^{II}–**H** base pairing.[24] Subsequent addition of EDTA to remove Cu^{II} ions resulted in the regeneration of the monomeric duplex (lane 4). In summary, assembly and disassembly of DNA duplexes driven by metal coordination was clearly demonstrated with the enzymatically synthesised ligand-bearing DNA duplexes. This strategy could be further applied for the metal-responsive assembly of DNA origami architectures, DNA-modified proteins and DNA-coated nanoparticles.[1,8]

In conclusion, we have succeeded in the enzymatic polymerisation of hydroxypyridone ligand-bearing nucleotide (d**H**TP) utilising template-independent TdT, which produced artificial DNA strands tailed with several **H** nucleotides at the 3′-end. The **H** ligand moieties were intact and formed metal-mediated **H**–Cu^{II}–**H** base pairs as was the case with chemically synthesised artificial DNAs. This TdT-catalysed reaction was further applied for the modification of DNA duplexes, by which metal-mediated DNA self-assembly was demonstrated. TdT-catalysed post-synthetic modification would be readily applied for DNA-modified nanomaterials and biomacromolecules. Accordingly, the methodology established here will allow for the development of DNA-based materials whose structures and functions can be regulated in response to metal coordination. It is worth to note that this method is presumably employed for other ligand-bearing nucleotides that form metallo-base pairs with different metal ions.[2,4,25] Recently, metallo-base pairs consisting of natural nucleosides, such as T–Hg^{II}–T, have been extensively used for developing metal-responsive functional DNAs.[2,26,27] Therefore, this study would open the door to future applications of a number of artificial metallo-base pairs in the filed of DNA nanotechnology, especially in the development of metal-ion sensors, metal-driven molecular machines and logic gates.[28] Enzymatic post-synthetic modification of other DNA structures with **H** nucleotides is now underway towards further application of the metal-mediated base pairing.

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