Functionalized DNA

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# Direct Polymerase Synthesis of Reactive Aldehyde-Functionalized DNA and Its Conjugation and Staining with Hydrazines**

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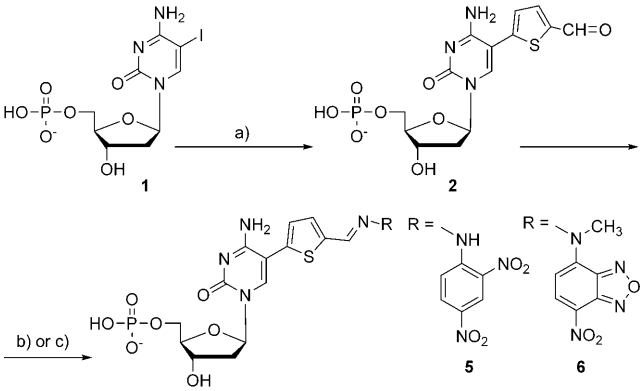
Apart from a wide range of novel applications of functionalized DNA in chemical biology, nanotechnology, and material sciences,[1] attachment of reactive functional groups to nucleic acids is needed for further transformations or bioconjugates. The introduction of alkyne, azide, or diene groups either by chemical phosphoramidite synthesis or by enzymatic polymerase synthesis has been achieved and the modified DNA was used for click-chemistry,[2,3] Staudinger ligation,[4] and Diels–Alder reactions.[5] An aldehyde functional group is a very attractive group because of its high and specific reactivity with diverse reagents. However, it was considered too reactive and fragile to be incorporated directly (chemically or enzymatically)[6] and the few successful examples were prepared indirectly by a click reaction with azide derivatives of reducing sugars,[3] or by introduction of 2,3-dihydroxypropyl or 3,4-dihydroxypyrrolidine moieties[7,8] and subsequent oxidative cleavage of the vicinal diols to (di)aldehydes. The syntheses of the nucleoside/nucleotide monomers were laborious multistep procedures and additional post-synthetic steps were required to release the aldehyde function in DNA.[7,8] Metallization[7] or interstrand cross-linking[8] were demonstrated to be very useful applications of aldehyde-modified oligonucleotides (ONs) or DNA. Therefore we decided to develop a simple and efficient direct protocol for construction of aldehyde-modified DNA by application of our two-step (cross-coupling polymerase incorporation) method.[9,10] In addition, we wished to develop a methodology for additional conjugation and staining of aldehyde-modified DNA by hydrazone formation.

The methodology of choice involved Suzuki cross-coupling of a halogenated nucleoside triphosphate (dNTP) with an aldehyde-containing boronic acid, and subsequent polymerase incorporation into DNA.[9,10] Furthermore, we wanted to develop a general methodology for hydrazone formation in aqueous media. To test the first and last steps of our proposed route, we performed the reactions on the model compound 5-iodo-dCMP (**1**; dCMP = 2′-deoxycytidine-5′-*O*-monophosphate). Commercially available 5-formylthiophene-2-boronic acid was selected as a suitable carrier for the aldehyde group, and its aqueous-phase cross-coupling with monophosphate **1** proceeded within 40 minutes and gave aldehyde-modified dCMP **2** in 50% yield (Scheme 1). The next task was the formation of the hydrazone species, which is usually only performed in dry organic solvents (owing to the formation of water in the reaction). To make the reaction amenable to aqueous conditions, we have adapted the protocol developed by Dawson and co-workers[11] for aqueous conjugation of peptides, which uses aqueous ammonium acetate and aniline to facilitate the condensation. To test the reactions with **2**, we selected two arylhydrazines (**3** and **4**) that are commonly used as aldehyde-specific dyes.[12,13] The reactions of aldehyde-nucleotide **2** with **3** or **4** proceeded at room temperature for approximately 20 hours and gave the corresponding orange (**5**) or violet (**6**) hydrazones, which were fully characterized (see the Supporting Information). As the formation of hydrazone in water is inherently a reversible reaction, the yields for the isolated products of 51 and 31%, respectively, were acceptable and useful.

The cross-coupling protocol was then applied in the reactions of iodinated dNTPs (**dC$^{I}$TP** and **dA$^{I}$TP**)[10,14] with 5-formylthiophene-2-boronic acid. The desired aldehyde-modified dNTPs (**dC$^{FT}$TP** and **dA$^{FT}$TP**) were isolated in 65 and 41% yields, respectively (Scheme 2).

Next we have tested the polymerase incorporation of **dC$^{FT}$TP** and **dA$^{FT}$TP** in primer extension (PEX) or polymerase chain reaction (PCR) experiments using several




***Scheme 1.*** Synthesis of aldehyde-modified cytidine monophosphate **2**, and subsequent synthesis of colored hydrazone-modified cytidine monophosphates **5** and **6**. Reaction conditions: a) 5-formylthiophene boronic acid, $Pd(OAc)_2$, TPPTS, $Cs_2CO_3$, ACN/$H_2O$ (1:2), 120 °C, 40 min, 50%; b) 2,4-dinitrophenylhydrazine (**3**), $NH_4OAc$, aniline, $H_2O$, RT, 21 h, 51%; c) 4-(1-methylhydrazino)-7-nitrobenzofurazane (**4**), $NH_4OAc$, aniline, $H_2O$, RT, 18 h, under Ar, 31%. TPPTS = 3,3′,3″-phosphinetriyltris(benzenesulfonic acid) trisodium salt.

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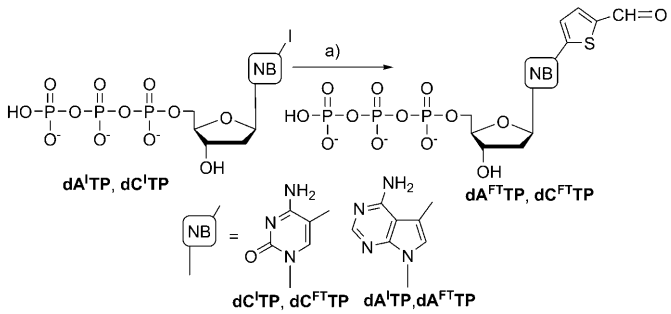


***Scheme 2.*** Synthesis of aldehyde-modified nucleoside triphosphates. Reaction conditions: a) 5-formylthiophene boronic acid, $Pd(OAc)_2$, TPPTS, $Cs_2CO_3$, ACN/$H_2O$ (1:2), 100 °C, 1 h, 65 and 41%.

polymerases.[15] The question was whether the polymerases would tolerate the presence of a reactive aldehyde group (which may even react with some functional groups of the protein, for example, the amino group of lysine) and incorporate the **dN$^{FT}$TP**s into DNA. The best results were obtained with Vent(exo$^-$) DNA polymerase, which readily and selectively incorporated both aldehyde-modified nucleotides by PEX—even incorporation into sequences containing multiple modifications (Figure 1a). DyNAzyme and Phusion polymerases were also efficient in their incorporation by PEX (see the Supporting Information). Apart from PAGE analysis with proper negative control experiments, the formation of aldehyde-modified ON was confirmed by MALDI analysis of 31-meric product containing four modified **A$^{FT}$** bases (see the Supporting Information). Thermal denaturation (melting temperature) of 31-meric DNA duplexes containing four **A$^{FT}$** or four **C$^{FT}$** bases showed

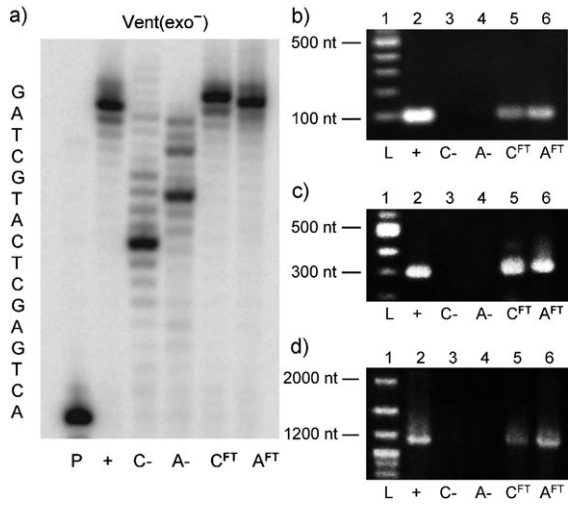


***Figure 1.*** a) Primer extension with Vent(exo$^-$) DNA polymerase. P: Primer (5′-$^{32}$P-end labeled primer-template); +: natural dNTPs; C-: dTTP, dATP, dGTP; A-: dTTP, dCTP, dGTP; C$^{FT}$: **dC$^{FT}$TP**, dTTP, dATP, dGTP; A$^{FT}$: **dA$^{FT}$TP**, dTTP, dCTP, dGTP. b) PCR synthesis of 98-mer by Pwo polymerase. Lane 1: ladder; lane 2 (+): natural dNTPs; lane 3 (C-): dTTP, dATP, dGTP; lane 4 (A-): dTTP, dCTP, dGTP; lane 5 (C$^{FT}$): **dC$^{FT}$TP**, dTTP, dATP, dGTP; lane 6 (A$^{FT}$): **dA$^{FT}$TP**, dTTP, dCTP, dGTP, c) PCR synthesis of 287-mer by KOD XL polymerase, d) PCR synthesis of 1162-mer by KOD XL polymerase.

increase in stability by 2.5 °C per modification (see the Supporting Information).

PCR is even more demanding in terms of efficiency and fidelity of incorporation and many previous modifications successful in PEX did not work in PCR.[10] However, the aldehyde-modified **dN$^{FT}$TP**s were excellent substrates even in PCR reactions. PCR with a 98-mer template (Figure 1b) proceeded smoothly with Pwo-polymerase-amplified DNA duplex products containing 37 (**A$^{FT}$**) or 34 (**C$^{FT}$**) aldehyde groups. Longer templates (287 and 1162 nt) were also subjected to PCR with **dN$^{FT}$TP**s. When using 287 nt template, Pwo polymerase did not work, while Vent(exo$^-$) gave only weak amplification. Only the use of KOD XL polymerase[7] gave efficient amplification with this template (Figure 1c). Therefore, the KOD XL polymerase was then used for successful PCR amplification of a long 1162 nt template (Figure 1d). In both cases, full-length products were detected with **dA$^{FT}$TP** being a somewhat better substrate that gave more efficient amplification compared to **dC$^{FT}$TP**. This outcome proves the possibility of these modified **dN$^{FT}$TP**s to be of general use in PCR.

The final goal was to apply the methodology for the synthesis and conjugation of aldehyde-modified DNA with hydrazines (Figure 2a). The 98-mer PCR product made from **dC$^{FT}$TP** (+ natural dATP, TTP, and dGTP) was treated with hydrazine **3** or **4**. The dinitrophenylhydrazone–DNA was stained to yellow, while the nitrobenzofurazanehydrazone–DNA was pink (Figure 2b). The corresponding unmodified PCR products treated with hydrazine **3** or **4**, as well as the aldehyde-DNA were colorless. After staining with hydrazines, the UV/Vis spectra of the modified PCR products were in full accord with the UV/Vis spectra of isolated and fully characterized nucleotide hydrazones **5** and **6** (Figure 2c and the Supporting Information). Recalculated from extinction coefficients and the Beer–Lambert law, the average number of hydrazone-modified cytosines was 19 (55%) and 16 (47%), for **B** and **C** (Figure 2) respectively, out of 34 aldehyde groups in the PCR product. This result is consistent with the yields of isolated hydrazones **5** and **6**. In addition, we have also used the 98-mer PCR-C$^{FT}$ and PCR-A$^{FT}$ products for Tollens reaction[7] to further prove the presence and reactivity of the aldehyde groups. In accord with the literature,[7] this reaction with aldehyde-modified DNA revealed a time-dependent increase of absorbance at 450 nm (see Figure S9 in the Supporting Information).

In conclusion, we have developed the first direct and efficient methodology for introduction of an aldehyde functional group to DNA in only two steps. The aldehyde group (attached through a thiophene moiety) withstands both Suzuki cross-coupling and polymerase incorporation. The aldehyde-modified dNTPs are excellent substrates for DNA polymerases and the aldehyde-nucleotides are readily incorporated by PEX or PCR to diverse ONs or DNA duplexes (even in a manifold fashion). We have also developed a simple aqueous conjugation with arylhydrazines and used this protocol for DNA staining. Further application of the (now) easily available aldehyde-modified DNA in conjugation with other useful hydrazines (fluorescent, redox active, etc.) or with diverse biomolecules are currently under way.

  

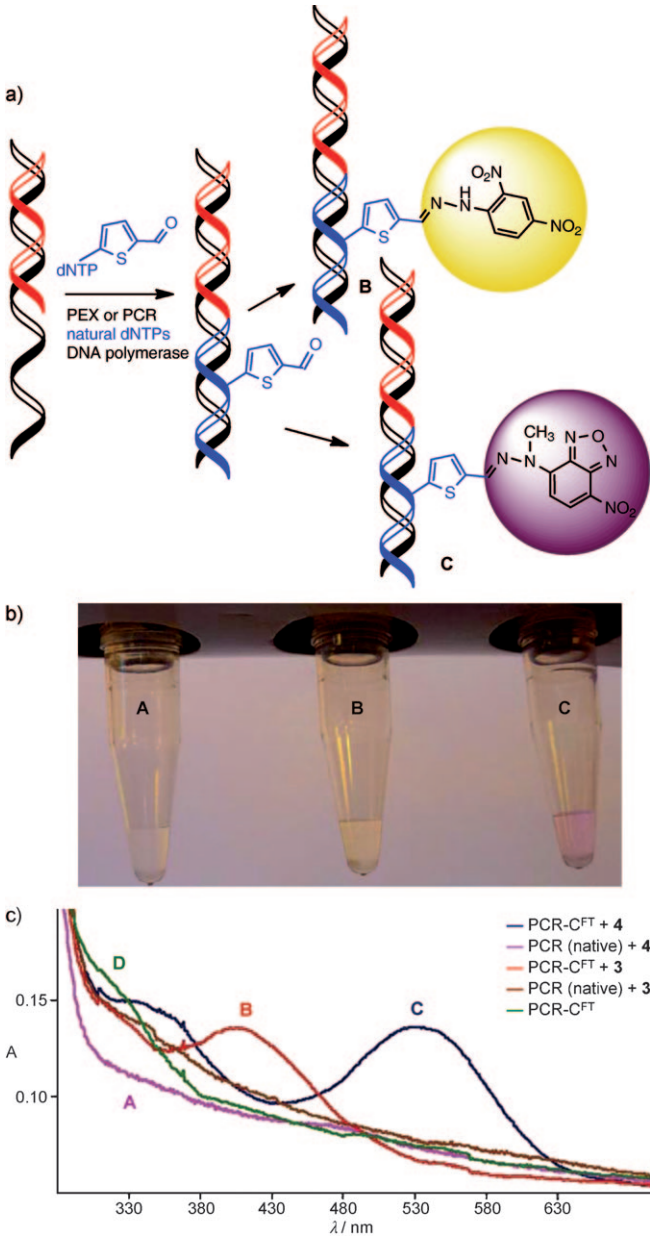


***Figure 2.*** a) Synthesis of aldehyde-functionalized DNA followed by staining with hydrazone formation. b) Color of PCR products after staining with hydrazines. A: PCR product from all natural dNTPs treated with **4** (colorless product); B: 98-mer PCR product from three natural dNTPs and **dC$^{FT}$TP** treated with **3** (yellow); C: 98-mer PCR product from three natural dNTPs and **dC$^{FT}$TP** treated with **4** (pink). c) UV/Vis spectra of unmodified and modified 98-mer PCR products without or after staining with hydrazines. A, B, and C, Spectra are of the same solutions as in b), D (green line) is the spectrum of C$^{TF}$-containing PCR product without staining (colorless).

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