DNA Modification

Generation and Enzymatic Amplification of High-Density Functionalized DNA Double Strands**

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Dedicated to Professor Julius Rebek, Jr. on the occasion of his 60th birthday

The specific pairing of DNA bases in double-stranded DNA (dsDNA) provides an intriguing rationale for the straightforward generation of molecular assemblies on the nanometer scale and has led to growing interest in the exploitation of DNA for nanotechnology, material science, computing, and biotechnology purposes.[1] The application of conventional DNA molecules for these purposes by utilization of simple Watson–Crick pairing has already led to considerable progress in the generation of an impressive variety of topological two- and three-dimensional geometries, nanomechanical devices, and supramolecular assemblies.[2] In addition, DNA exhibits unique template properties which allows for its enzymatic replication and amplification by DNA polymerases and enables the development of sensors based on nucleic acids, therapeutics, and catalysts by in vitro evolution.[3]

The scope of applications of dsDNA in nanotechnology would be greatly expanded if DNA molecules could be modified in a base-specific fashion with additional chemical functionalities that could be employed for other functions and interactions apart from Watson–Crick pairing. For maximal flexibility, it would be important to develop strategies that allow for the modification of as many base positions as possible within a given DNA strand without interfering with base pairing. To achieve replication and amplification of such nanostructured systems DNA polymerases that tolerate a broad range of substrates and templates would have to be generated by in vitro evolution.[4] Alternatively, reaction conditions would have to be found that enable enzymes to process unnatural templates and monomers that are usually not replicated. This would open up the possibility to synthesize longer DNA constructs (>100 bp) which are usually inaccessible synthetically. As a consequence, a larger sequence space of functionalized DNA molecules, including amplifiable libraries for in vitro selection experiments, could be generated faster and more easily.

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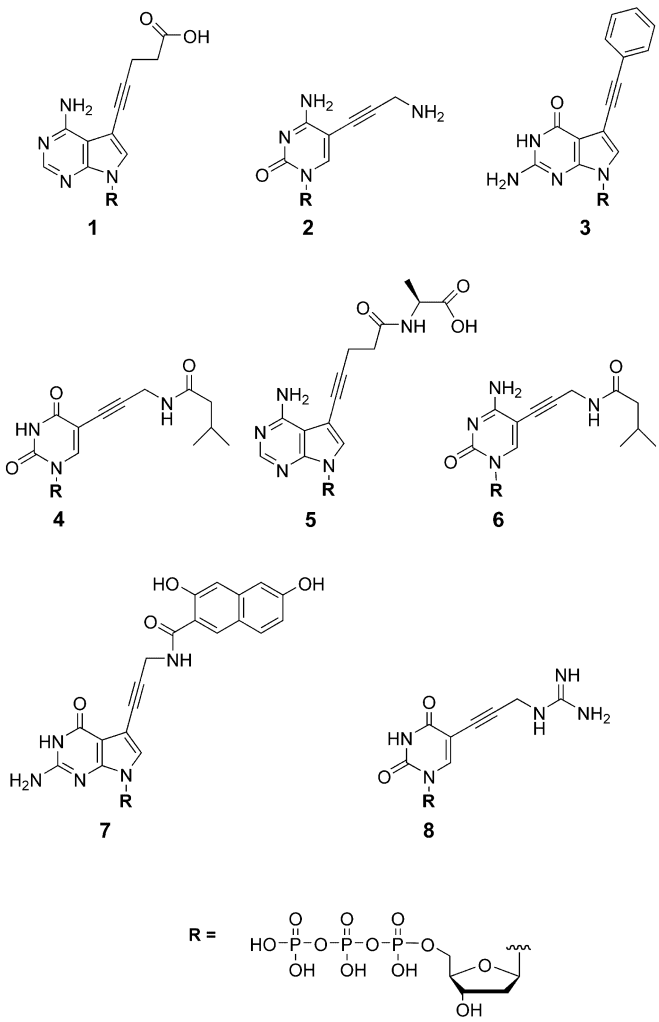
A series of chemically modified 2′-deoxynucleoside triphosphates has already been synthesized, and the template-directed enzymatic polymerization of up to two different modified nucleotides was achieved by primer extension or polymerase chain reaction (PCR).[5,6] Single-stranded high-density functionalized DNA (fDNA) in which every base is modified with additional functionality can be generated by enzymatic primer extension of base-modified deoxynucleoside triphosphates (dNTPs) using conventional DNA as a template.[7,8] Single-stranded fDNAs containing residues **1**–**3** and **8** (Scheme 1) can in turn serve as templates in PCR



Scheme 1. Chemical structures of base-modified 2′-deoxynucleoside-5′-*O*-triphosphates.

reactions with natural nucleotides.[7] However, the utilization of a high-density fDNA template for the enzymatic synthesis of the corresponding fDNA counterstrand has so far been unsuccessful. For this to occur, the polymerase would not only have to recognize the functionalized DNA strand as a template, but would also have to be able to incorporate modified bases—according to the directions of the template—opposite a certain base modification. The ability to do so would not only allow for the straightforward synthesis of

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fDNA double strands and their enzymatic amplification, but would also enable the introduction of even further chemical diversity into the counterstrand. Consequently, both strands could be decorated with different chemical groups sequence-specifically. Functionalized dsDNA modified at high density in such a way would enable the directed assembly of completely novel supramolecular structures which could not be accessed using conventional dsDNA.

Herein we describe the first example of an enzymatically synthesized fDNA double strand in which all four nucleobases in each strand are substituted with various base analogues, thus allowing the introduction of up to eight different modifications into one dsDNA. The dNTP derivatives used (Scheme 1) cover a broad range of different functionalities, such as aromatic (**3**, **7**), basic (**2**, **8**), acidic (**1**, **5**), and lipophilic residues (**4**, **6**).[9] Furthermore, we have established a screen to identify and optimize conditions that enable the direct amplification of fully modified fDNA by PCR.

We synthesized a fully functionalized fDNA template enzymatically under similar conditions as described before from a 79 nucleotide 5′-biotinylated DNA template **M79** using dNTPs **1**–**4**.[7,10] The dNTPs **1**–**4** proved to be good substrates for the Vent(exo-), and Pwo DNA polymerase and replaced their natural counterparts sequence specifically (data not shown). We then tested whether **fM79** could serve as a template in standard primer extensions in the presence of dNTPs **1–4** using Pwo DNA polymerase.

As shown in Figure 1 a, full-length double-stranded fDNA could be obtained (lane 5), whereas omitting one of the four modified dNTPs resulted in premature termination of the counterstrand synthesis (lanes 1–4). A primer extension using nonfunctionalized **M79** template in the presence of natural dNTPs served as a size marker (lane M). Figure 1 b shows the same set of experiments, but this time a new set of modified dNTPs **5**–**8** (Scheme 1) was used that bear chemical functionalities different from those in the **fM79** template modified with **1**–**4**. Again, the full-length primer extension product was obtained (lane 10). This product represents the first example of a high-density functionalized dsDNA in which each strand contains a different set of modifications in one fDNA double strand. Furthermore, these data show that Pwo DNA polymerase not only accepts a high-density functionalized DNA single strand as a template, but is also capable of template-directed incorporation of all four modified dNTPs opposite a modified base.

We next investigated whether the conditions under which primer extension could be achieved would also allow for amplification of double-stranded fDNA under PCR conditions. PCR amplification under standard buffer conditions with the **fM79** template and the modified dNTPs **1**–**4** did not result in amplification of product with the desired length (Figure 2 b, lane 1a). Therefore, we tested different concen-

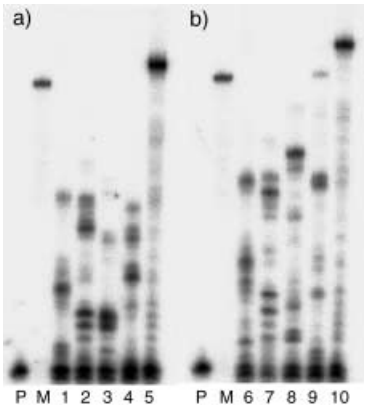

Figure 1. Primer extension experiments with ^{32}P-labeled primer **P5**′ and template **fM79**, modified dNTPs, and Pwo polymerase, with analysis by gel electrophoresis (8% denaturing polyacrylamide). a) Lanes: P: primer; M: primer extension with four natural dNTPs and **M79** as template; 1: reactions with modified nucleotides **2**–**4**; 2: **1**, **3**, and **4**; 3: **1**, **2**, and **4**; 4: **1**–**3**; 5: **1**–**4**. b) Lanes P: primer; M: primer extension with four natural dNTPs and **M79** as template; 6: **6**–**8**; 7: **5**, **7**, and **8**; 8: **5**, **6**, and **8**; 9: **5**–**7**; 10: **5**–**8**.

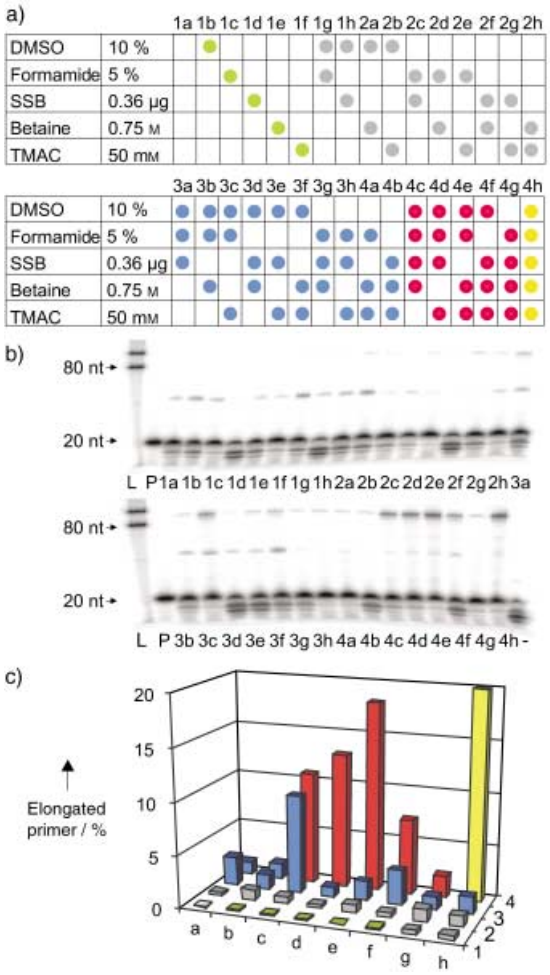

Figure 2. Combinatorial screen of 32 different buffer conditions for the PCR. The PCR experiments included ^{32}P-labeled primer **P5**′, dNTPs **1**–**4**, and template **fM79.** Lane numbers 1a–4h refer to the different combinations of additives. The PCR products were analyzed by gel electrophoresis (10% denaturing polyacrylamide). a) Reaction buffers. Dots indicate the presence of the respective additive in the PCR buffer. b) Lanes L: length marker; P: primer; 1a–4h: PCR reactions with the respective buffer conditions (a); (−): control without template. c) Quantification of the PCR products by PhosphorImaging as the percentage of the total radioactivity.

trations of various additives, such as tetramethylammonium chloride (TMAC), dimethyl sulfoxide (DMSO), formamide, *E. coli* single-strand binding protein (SSB), and betaine, which are known to have a positive effect on PCR reactions using the highly thermostable Pwo DNA polymerase. No significant improvement was observed when either of these components was added to the PCR reactions alone (Figure 2b, lanes 1b-f).

We performed a combinatorial screen of all possible permutations of combinations of the five additives to investigate the possibility that combinations of these additives would improve PCR amplification. Figure 2a shows all the various compositions of additives. The quantified results are presented in Figure 2b and c. As a positive control of polymerase activity we tested the same buffer conditions using natural dNTPs as the monomer source (see Supporting Information).

Indeed, considerable amounts of full-length high-density functionalized dsDNA PCR products could be obtained with certain combinations of at least three different additives, although in the "three-component-set" (Figure 2, 3a–4b, blue dots) only combination 3c led to significant amplification. Combinations of four different components significantly improved the amount of PCR product, except for 4f and 4g (Figure 2) in which either DMSO or formamide were omitted. Addition of all five components (4h) did not significantly improve the results obtained with combination 4e, which led to the best result in the "four-component set" (4c–4g, red dots). The amount of high-density modified dsDNA obtained corresponds to about 30% of the amount obtained in the control experiment in which the natural dNTPs were used exclusively with the same number of cycles (see Supporting Information). These data thus allow the conditions to be defined that enable PCR amplification of high-density functionalized double-stranded fDNA using functionalized dNTPs as the only monomer source.

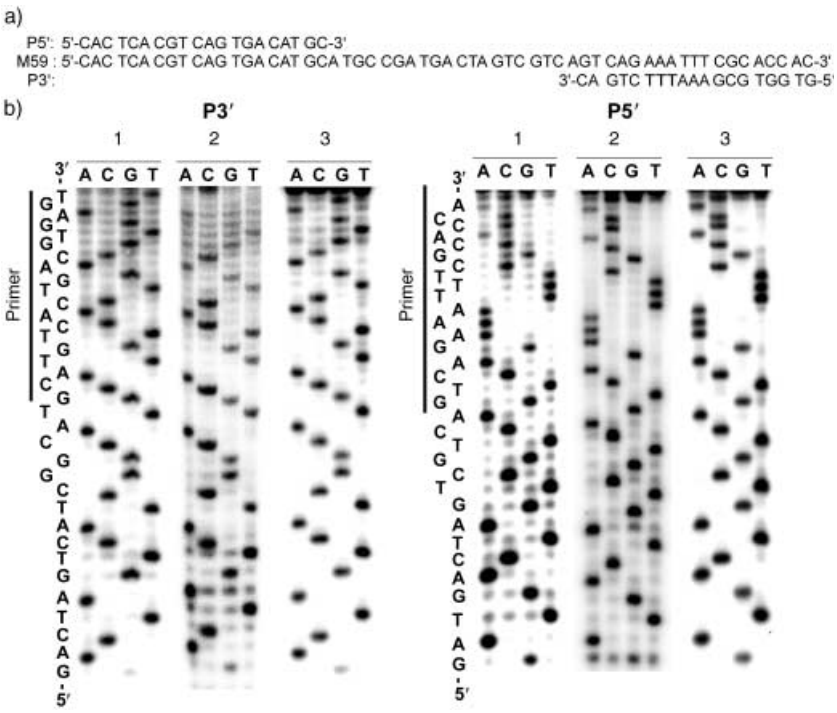

Figure 3. Autoradiograms of the Sanger dideoxy sequencing reaction with T7 DNA polymerase. a) Sequencing primers **P3′**, **P5′**, and template **M59**. b) 1. Sequencing of the original template **M59**; 2. sequencing of fully functionalized DNA **fM59**, which was generated by PCR with modified dNTPs **1**–**4**. 3. Sequencing of a PCR product derived from **fM59** as a template and natural dNTPs. Sequences were read in both directions with primers **P3′** and **P5′**. The sequencing patterns from the original template **M59** (1), the direct sequencing of fDNA **fM59** (2), and of the natural PCR product derived from functionalized **fM59** as template (3) are indistinguishable.

To investigate the accuracy of the incorporation of the modified nucleotides and the PCR amplification of fDNA under conditions 4e (Figure 2) with Pwo DNA polymerase we synthesized a fully modified double-stranded fDNA in 20 PCR cycles from a DNA template **M59** (Figure 3a) and dNTPs **1**–**4** (Scheme 1). The strands were separated (see Supporting Information) and sequenced using T7 DNA polymerase by the dideoxy method developed by Sanger (Figure 3b, 2). To verify that the additional bands did not originate from mis-incorporation of modified nucleotides we amplified the modified template **fM59** with natural dNTPs and sequenced the resulting natural dsDNA (Figure 3b, 3). As a control we also amplified and sequenced the original template **M59** (Figure 3b, 1). Comparison of the sequencing patterns shows that the sequence information is conserved during PCR amplification with **1**–**4** and that the incorporation of the modified nucleotides is sequence-specific within the accuracy of a sequencing reaction. Interestingly, similar to Pwo polymerase, T7 DNA polymerase is also able to read modified DNA strands correctly.

Furthermore, we examined whether the high-density modification of a dsDNA affects the conformation relative to that obtained with the corresponding nonmodified dsDNA. Indeed, comparison of the CD spectra of both species revealed that a natural dsDNA version of **M59** showed a typical B-DNA spectrum (Figure 4, ▲), while functionalized double-stranded DNA of the same sequence **fM59** (Figure 4, □) showed the same inversion of the CD spectrum as observed originally for Z-DNA under the same conditions.[11] A similar inversion of the CD spectra was observed by Brakmann and Löbermann when all the pyrimidine residues of one strand were substituted with fluorescently labeled analogues.[6] However, the wavelengths observed in the CD spectra of fDNA **fM59** are significantly shifted compared to those with unmodified Z-DNA motifs, which might be a result of the altered polarizability of the nucleobases. Similar wavelength shifts were found in short DNA duplexes that contained sporadic alkinyl-modified purines and pyrimidines.[12] The CD spectra obtained in this study showed more similarity to B-DNA, in contrast to the fully modified

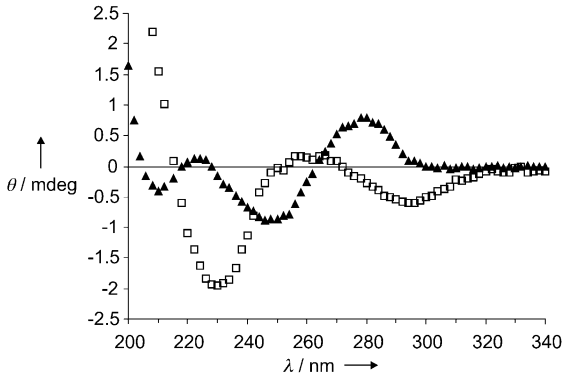

Figure 4. CD spectra of natural, unmodified dsDNA **M59** (▲) and dsDNA **fM59** fully modified with dNTPs **1**–**4** (□).

sequence investigated here, which suggests that the conformation of the fully modified dsDNA changes from a right-handed B-type to a left-handed Z-type DNA.

Interestingly, the visualization and quantification of the double-stranded fDNA products shown in Figure 2b necessitated the use of a radiolabeled primer because these products could only be slightly stained with ethidium bromide. Presumably, the presence of the modifications at the 5-position of the pyrimidines and at the 7-position of the purines not only leads to a conformational change but also to sterically hindering intercalation.

In conclusion, this study demonstrates for the first time that high-density DNA double strands can be generated enzymatically in a template-directed fashion and can be amplified by PCR under certain conditions. Remarkably, a natural DNA polymerase is capable of selecting the correct base-modified nucleoside triphosphate opposite a modification in the template and of catalyzing its incorporation into the growing counterstrand. The incorporation of chemical functionalities into a DNA double strand at high-density with nanoscale resolution opens up the possibility to program higher-order dsDNA architectures, assembled through base pairing, with additional functionalities. Thus, this approach offers significant potential to facilitate the construction of complex nanoscale objects and materials based on DNA.

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