DNA Nanostructures

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Thermostable Branched DNA Nanostructures as Modular Primers for Polymerase Chain Reaction**

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The extraordinary controllability of synthetic DNA nanostructures offers great potential for nanotechnology and biotechnology,[1–5] with applications including self-assembly,[6–11] sensing,[12–18] and DNA-based materials.[19–22] However, DNA nanostructures are unstable at high temperatures because of the inherent thermal instability of base pairing, which limits their practical application. Recent work has demonstrated the use of psoralen, a naturally occurring reagent that intercalates and cross-links DNA,[23] to create DNA origami structures with enhanced thermal stability.[24,25] In addition, other thermostable bonds have been introduced in the form of DNA/organic molecule hybrids to construct DNA structures and networks.[26–28]

Herein, we present a general approach using our previously reported branched DNA building blocks[29,30] combined with psoralen cross-linking to produce thermostable branched DNA nanostructures. These nanostructures can withstand denaturing conditions without disruption of their integrity. Importantly, this system is extremely modular. Simple nanostructures can be combined in a controlled fashion (by enzymatic ligation) to form increasingly complex nanostructures with predesigned labeling and functions. This unique modularity enabled us to utilize thermostable branched DNA nanostructures as modular primers in polymerase chain reaction (PCR). Our PCR with modular primers maintains the controllability of branched DNA nanostructures, while preserving the biological function of the resulting PCR products. Furthermore, we demonstrate the novelty and utility of PCR with modular primers through the realization of multifunctional labeling for detection, synthesis of branched PCR products, and hydrogel formation, none of which can be achieved by the use of conventional linear primers.

Our branched thermostable DNA structures were formed by a two-step process (Figure 1a). In the first step, we synthesized Y-shaped DNA following our previously reported protocol.[29] Briefly, three single-stranded DNA molecules were rationally designed with specific sequences such that each was partially complementary to another, resulting in self-annealed, branched, Y-shaped DNA. In the second step, the structures were incubated with psoralen and briefly exposed to UV illumination. DNA sequences were deliberately designed to obtain a high proportion of interstrand cross-linking sites during this psoralen treatment step (Supporting Information, Figure S1). After this simple treatment, the branched DNA nanostructures were thermostable and remained intact even under denaturing conditions. To implement our structures in PCR, we simply used our modular primers in place of the standard linear primers (Figure 1b). During the denaturing phase of PCR, the linear template was denatured while the modular primers remained intact as

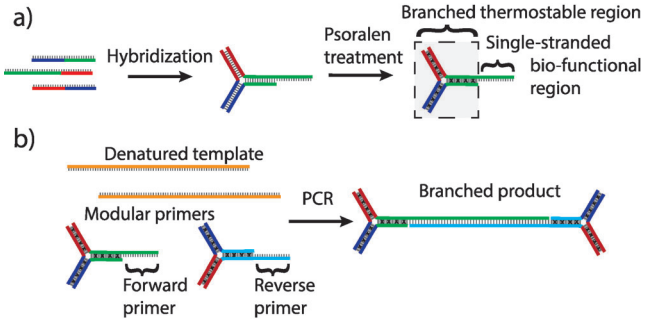

Figure 1. Illustration of cross-linking and subsequent primer extension by PCR. a) Single-stranded oligonucleotides hybridize to form branched DNA nanostructures through DNA base pairing. Nanostructures are treated with psoralen to cause covalent interstrand cross-linking. Resulting structures are thermostable and will remain intact under denaturing conditions. b) Cross-linked products can be used in PCR as modular primers in place of the conventional linear primers, thus resulting in branched PCR products.

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a result of cross-linking with psoralen. During the annealing and extension phases, the modular primers hybridized to the template and were extended by the polymerase, resulting in a dumbbell-shaped product. Exponential amplification occurred as usual, except that products were branched.

Prior to carrying out PCR, we first confirmed the thermal stability of the cross-linked branch by melting curve analysis. The non-cross-linked branch exhibited a melting transition at the normal T_m of 55 °C (Figure 2a). However, no melting

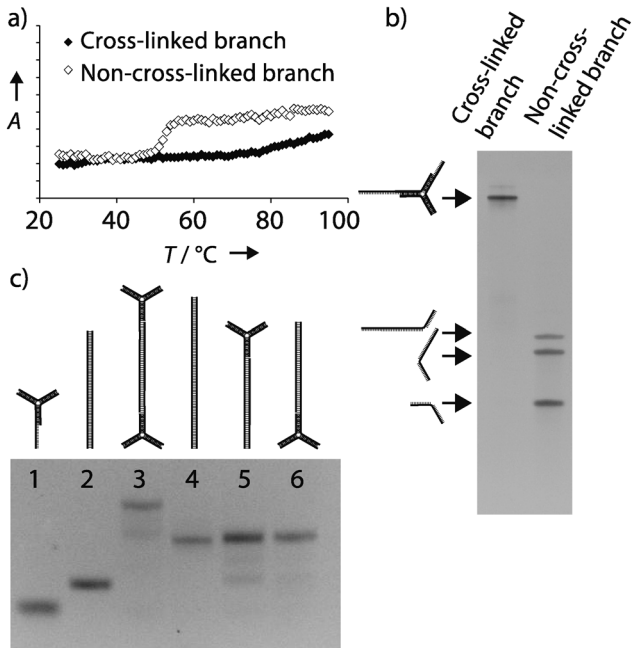

Figure 2. a) Melting curve of cross-linked branched DNA (black data points), and non-cross-linked branched DNA (white data points). b) Denaturing gel electrophoresis of cross-linked branched DNA (upper arrow), and non-cross-linked branched DNA (lower arrows). c) Non-denaturing gel electrophoresis of PCR products resulting from modular primers. Lane 1: Y-shaped modular primer. Lane 2: PCR product using conventional linear primers. Lane 3: PCR product using Y-shaped modular primers, resulting in branched DNA product. Lane 4: PCR product using non-cross-linked branched DNA. Branched DNA is denatured during thermal cycling, resulting in non-branched products. Lane 5: PCR product, in which only the forward linear primer was replaced with a modular primer. Lane 6: PCR product, in which only the reverse linear primer was replaced with a modular primer.

transition was evident for cross-linked branches up to 95 °C, although absorbance increased slightly at high temperatures because of increased breathing within non-cross-linked regions of the double-stranded DNA. In addition, we further tested the stability of the cross-linked branch in the presence of a strong denaturant (7 M urea) in denaturing gel electrophoresis. The cross-linked branch remained intact (Figure 2b, upper arrow), while the non-cross-linked branch was completely denatured into its constituent single-stranded DNA (Figure 2b, lower arrows). The resulting yield of cross-linked product was approximately 100% (no bands corresponding to single-stranded oligonucleotides in the first lane). To further test the stability of more complex DNA nanostructures using our approach, we ligated simple Y- and X-shaped DNA to form a variety of branched DNA nanostructures, and tested their stability using denaturing gel electrophoresis and thermal cycling (Supporting Information, Figures S2 and S3).

After confirming the stability of the cross-linked branched DNA, we carried out PCR with our modular primers in place of the conventional linear primers. Branched PCR products were observed in these samples, which confirmed that the cross-linking treatment did not interfere with the polymerase activity (Figure 2c). Lane 3 confirmed that virtually all primers were used in the PCR reaction, and we further used gel densitometry to estimate that the yield of the dumbbell-shaped PCR product was more than 85%. We also designed more complex modular primers, which showed similar compatibility with PCR (Supporting Information, Figure S4). These results demonstrated the versatility of our modular primer system, which affords the flexibility to realize a variety of PCR processes.

In conventional PCR, each primer can be labeled with only one fluorophore or functional moiety. In contrast, our modular primers can be labeled with multiple moieties in a controllable fashion, taking advantage of the anisotropy of branched DNA nanostructures. In our previous work, we developed a "DNA nanobarcode" system, which used branched DNA with specific color ratios of fluorophores to correspond to specific pathogen targets.[31] Using modular primers in combination with the DNA nanobarcode concept, we carried out multiplexed PCR with three different target DNA sequences (Figure 3a). In the multiplexed PCR, each PCR product was labeled with a specific color ratio (Figure 3b). This approach enables the simultaneous amplification and labeling of target sequences in a single step.

Conventional linear primers allow polymerization to extend in only one direction, resulting in a linear product. The use of modular primers enabled polymerization in multiple directions, which resulted in multiple PCR products attached to a single branched nanostructure (Figure 3c). The PCR products were a mixture of one-, two-, and three-arm products, with a distribution that depended on the length of template used (Supporting Information, Figure S5). For the case of a 1 kB template, the yield of the three-arm product was approximately 10%. These branched PCR products were further confirmed by AFM single-molecule imaging,[32] which clearly indicated that multiple products were amplified directly from a Y-shaped branch (Figure 3d).

Further utilizing our modular primers, we linked multiple Y-shaped DNA branches together to form an interlinked three-dimensional network, which resulted in a bulk hydrogel. Previous works have demonstrated formation of DNA hydrogels using ligation, polymerization, hybridization, and specific binding of DNA motifs.[20–22,33] To the best of our knowledge, this is the first time a bulk hydrogel has been produced entirely from PCR processes. More specifically, we designed two modular primers: one contained three linear forward primers and the other contained three linear reverse primers (red and green, respectively, Figure 3e). During the PCR process, these primers were extended to form connections among the Y-shaped DNA branches, forming a networked structure (Figure 3e–g; Supporting Information, Fig-

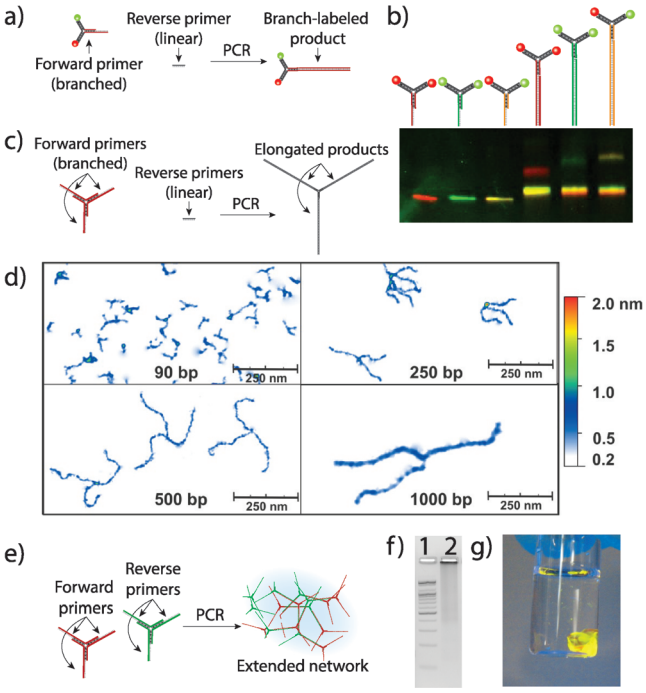

Figure 3. a) Modular primers were prepared with multiple labels. b) Gel electrophoresis demonstrating multiplexed PCR using multifunctional modular primers that were prepared with a combination of FAM (green) and Cy5 (red) fluorophores. Lanes 1–3: Three modular primers individually, showing the specific color ratio for each primer. Lanes 4–6: PCR reactions containing all primers yield PCR product with specific color ratio (red, green, and yellow, respectively) attached to the corresponding product. Lower bands (yellow is the result of a mixture of red and green fluorophores) in lanes 4–6 correspond to mixtures of non-extended primers which did not participate in the PCR because their corresponding target was not present. c) Branched forward primers and linear reverse primers yield three elongated products attached to a single Y-shaped branch. d) AFM images of branched structures extended to PCR products. e) Formation of DNA hydrogel by PCR. f) PCR-generated hydrogel remains trapped in well during gel electrophoresis. Lane 1: 100 bp DNA ladder. Lane 2: branched PCR product. g) PCR products were concentrated and annealed to form a DNA hydrogel, which was stained with GelGreen and photographed in solution under UV illumination.

ure S6), in which the Y-shaped DNA branches acted as cross-linking points within the network. After concentration of the PCR products, this network formed a DNA hydrogel. In contrast to previous works on DNA hydrogels, the use of PCR enables genes to be amplified from low concentrations while being simultaneously incorporated into the gel network. We incorporated the gene for a reporter protein, green fluorescent protein (GFP), into the gel by PCR, and we observed substantial production of GFP upon carrying out in vitro expression (Supporting Information, Figure S8). This approach is analogous to our previous work on cell-free expression with DNA hydrogels, but uses PCR rather than ligation as the mechanism for introducing genes into the gel.[34,35] This confirmed that PCR with modular primers can create a generic structure while simultaneously incorporating the genetic functions of the resulting PCR products.

In conclusion, we have developed thermostable branched DNA nanostructures that can be utilized as modular primers for PCR, allowing us to take advantage of both the self-assembly and biological capabilities of DNA in a single PCR process. This enabled the attachment of multiple moieties, such as fluorescence dyes and genes, onto a single branched PCR product, as well as the production of a PCR-generated DNA hydrogel. We expect this modular primer system to be further expanded to utilize PCR with more complicated DNA-based nanoarchitectures, such as DNA origami, DNA canvas, or molecular devices,[6,7] and we envision that thermostable DNA nanostructures will have great utility for DNA-based materials in practical applications.

Experimental Section

Branched DNA structures were synthesized by mixing the same molar amount of corresponding oligonucleotide strands. Samples were cross-linked by mixing with trioxsalen at a molar ratio of 1:1 between psoralen and DNA base pair, diluted in aqueous NaCl solution (50 mM) to a final volume of 500 μL and transferred to a 24-well plate for cross-linking. Exposure to UV light was performed using a 365 nm UV-A lamp at approximately 2.5 $mW\,cm^{-2}$ for 15 min at room temperature. The thermostability of products was confirmed with 15% Ready Gel TBE-urea polyacrylamide denaturing gel (BioRad). All PCR reactions were carried out using 2× Taq Master Mix (New England Biolabs) according to standard protocols, except that conventional linear primers were replaced by branched thermostable structures.

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