Generic Technique to Generate Large Branched DNA Complexes

Paul Tosch,[†] Christoph Wälti,*[,†] Anton P. J. Middelberg,[‡] and A. Giles Davies[†]

School of Electronic and Electrical Engineering, University of Leeds, Leeds LS2 9JT, United Kingdom, and Centre for Biomolecular Engineering, University of Queensland, St. Lucia, QLD 4072, Australia

Received November 22, 2005; Revised Manuscript Received January 31, 2006

The inherent self-recognition properties of DNA have led to its use as a scaffold for various nanotechnology self-assembly applications, with macromolecular complexes, metallic and semiconducting nanoparticles, proteins, inter alia, being assembled onto a designed DNA scaffold. Such structures may typically comprise a number of DNA molecules organized into macromolecules. Many studies have used synthetic methods to produce the constituent DNA molecules, but this typically constrains the molecules to be no longer than around 100 base pairs (30 nm). However, applications that require larger self-assembling DNA complexes, several tens of nanometers or more, need to be generated by other techniques. Here, we present a generic technique to generate large linear, branched, and/or circular DNA macromolecular complexes. The effectiveness of this technique is demonstrated here by the use of Lambda Bacteriophage DNA as a template to generate single- and double-branched DNA structures approximately 120 nm in size.

Introduction

A substantial part of current research in the field of molecular nanotechnology has focused on using biological molecules as scaffolds in the construction of functional devices. This is because many biological molecules are known to self-assemble into complex structures, using sophisticated molecular lock-and-key recognition processes. In particular, the inherent selective self-assembly and molecular recognition properties of DNA make this molecule an obvious candidate to construct scaffolds of a defined topology onto which other macromolecules can be assembled in a specific manner.[1] For example, proteins have been linked via DNA aptamers to specific sites on DNA molecules arranged into triple-crossover tiles, resulting in a highly ordered, self-assembled protein-array;[2] metallic nanowires have been formed by deposition of Ag,[3] Au,[4,5] Pt,[6] and Pd,[7] inter alia, onto DNA scaffolds; and Cu^{2+} ions have been assembled into regular arrays using DNA as a scaffold, resulting in magnetic chains.[8] Furthermore, the ability to generate branched scaffolds which could then be integrated into even larger structures could be particularly advantageous.[1,9]

In its most common form, DNA is a linear molecule and therefore not directly suited for the assembly of branched and interlinked scaffolds. However, a number of researchers have fabricated branched DNA structures from synthetic DNA molecules, including three-[10,11] and four-arm structures[12] and Borromean rings.[13] These branched DNA structures were used to assemble, for example, two-dimensional lattices[14] and DNA cubes.[15] Other work has combined plasmid-derived DNA with synthetic sequences to form DNA octahedron complexes spontaneously.[16] Yan et al. used such two-dimensional lattices assembled from branched DNA structures as a scaffold to generate ordered protein arrays.[17] These approaches elegantly show how synthetic DNA can easily be manipulated into self-assembling nonlinear complexes. However, the use of synthetic DNA inevitably limits the size of the constituent molecules and, therefore, the range of applications. For example, any program in which it is desired to integrate the DNA assembly into structures patterned by state-of-the-art nanofabrication tools will require DNA scaffolds several tens of nanometers in dimension.

Several studies have addressed this problem using different approaches. Keren et al. used RecA proteins to promote stable three-arm junction formation in homologous DNA fragments.[18] Cooper and Hagerman studied the structure and function of Holiday junctions by using synthetic DNA to construct four-arm junctions which were extended by plasmid-derived DNA via ligation.[19] Large tree-like or dendrimer-like structures have also been generated by using DNA constructs which are assembled in a stepwise fashion using synthetic oligonucleotides.[20,21] Similarly, chemical dendrimers which consist of small multi-branched molecules with synthetic single-stranded DNA oligonucleotides attached to the junction arms have been used as building blocks to form large dendrimeric structures by utilizing the complementarity of the oligonucleotides.[22] However, for applications where large branched complexes made exclusively from DNA are required, for example DNA scaffolds, none of these methods can be used. They rely on synthetic DNA to introduce branching, on subsequent extension of the junction arms by ligation, or on junction-formation promoting proteins. To tackle this problem and to construct branched pure DNA complexes, assembly methods similar to what is generally used to fabricate small DNA junctions, i.e., self-assemble appropriate single stranded DNA fragments into the desired geometry, could be employed. Therefore, a method of generating large single stranded DNA molecules of appropriate sequence is needed and, in principle, enzymatic methods can be exploited. Using this approach, the size of the branched higher order DNA complexes is limited only by the ability of the enzymes used to process the constituent linear DNA molecules. A further advantage of using DNA chemistry in this way is the large enzymatic toolkit available to manipulate the molecules. This naturally occurring

* Corresponding author. E-mail: c.walti@leeds.ac.uk.
[†] University of Leeds.
[‡] University of Queensland.

10.1021/bm050890g CCC: $33.50
Published on Web 02/25/2006

toolkit is exploited in molecular biology to manipulate linear DNA in vitro, however, in vitro enzymatic generation of nonlinear DNA remains largely unexplored.

In this letter we report and discuss a new generic enzymatic technique to generate large, branched DNA complexes, and in this context, we will demonstrate the assembly of a three-arm DNA junction with unique single stranded overhangs on all arms. The technique enables, for the first time, the assembly of complexes of several hundreds of nanometers in size consisting exclusively of native DNA without using difficult ligation processes to stitch the complex together.

The technique is based on the concept of using enzymatic methods to generate large single stranded DNA components each comprising two fragments with distinct base sequences: an upstream fragment and a downstream reverse complement fragment. Each fragment is complementary to a fragment of another single stranded component, thus allowing for the formation of the whole DNA complex by self-assembly.

Materials and Methods

Synthetic Oligonucleotides. A comprehensive list of oligonucleotide sequences used to generate and ligate the fragments for the single branched DNA complex (components α, *β*, *γ*) and the double branched DNA complex (components α, *δ*, *γ*, and *ϵ*) is given in the Supporting Information. All oligonucleotides were supplied desalted, from either Operon Biotechnologies or Sigma Genosys.

Bacterial Plasmid Generation. Individual component fragments were PCR amplified using Eppendorf Hotmaster Taq and digested with the appropriate restriction enzyme (see primer list in the Supporting Information). Digested fragments were ligated into pGEM-T easy (Promega) and transformed into JM109 (Promega). Candidate bacterial colonies were blue/white screened for an insert of the appropriate size and sequenced to ensure they contained the required template.

T7 Gene 6 Exonuclease Digestion. Using the appropriate plasmid template, components were PCR amplified using an upstream primer containing five phosphorothioates on the 5′ end (see the Supporting Information for a list). The purified PCR products were digested using 20 units of T7 Exonuclease (New England Biolabs, M0263) for 120 min at 37 °C. Digested products were purified before annealing with Qiaquick columns (Qiagen) using PB buffer to bind the single stranded DNA to the column.

Annealing of Single Stranded Components. Purified single stranded components α, *β*, *γ* (α, *δ*, *γ*, and *ϵ* for the double branched complex) were heated in a Mastercycler EP S (Eppendorf, Germany) with a heated lid, to 95 °C for 10 min, and allowed to cool over 15 h using a program written to follow the temperature decay curve shown in the Supporting Information, Figure S2.

Atomic Force Microscopy. DNA was deposited onto mica substrates (Aztech Trading) as follows. Freshly cleaved mica was washed with a drop of deionized water (18.2 MΩ cm, Millipore) for 10 s, and excess water was removed. The mica was then covered in 10 mM $MgCl_2$ for 10 s, with the excess removed. Assembled DNA complexes at approximately 1 ng/*μ*L in 10 mM $MgCl_2$ were then allowed to adsorb onto the surface for 5 min. The mica substrate was washed twice with deionized water for 10 s and blow-dried under nitrogen. Imaging was performed using tapping mode AFM (DI Multimode AFM, Veeco, USA) under air, using etched silicon tips (OTESPA, Veeco, USA). Length measurements of the imaged structures were performed using the ImageJ image analysis software.[23]

Results and Discussion

Branched DNA Design. The schematic design for our three-arm DNA complex is shown in Figure 1a and comprises three

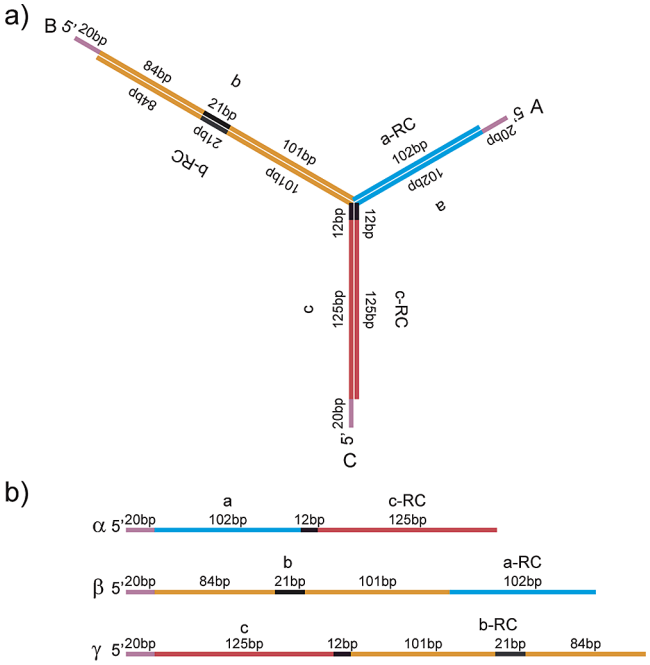

Figure 1. Design schematic for three-way complex. (a) Schematic of final assembled three-way complex after annealing. (b) Three single stranded components. Each component contains two regions which are complementary to regions of a neighboring component (indicated by blue, red, and orange). In addition each component has an upstream 20bp region which leads to a single stranded overhang in the final complex.

arms, A, B, and C, each composed of an upstream fragment (a, b, and c) and a downstream fragment (a-RC, b-RC, and c-RC). The individual arms A, B, and C were taken from suitable regions of lambda-Bacteriophage DNA (Sigma D0144). The only restrictions imposed on the individual regions was the required length of the arm and that they do not contain recognition sites for a certain set of enzymes which will subsequently be used for modifications of the assembled complex and for checksum tests. For the three-arm complex, three components are required, designed such that each fragment of a given component will recognize an appropriate fragment of another component. The individual components for the three-arm complex are shown schematically in Figure 1b (components α, *β*, *γ*). As can be seen from Figure 1, each component contains two fragments which will each form one-half of an arm of the DNA complex. In the case of the three-arm complex discussed here, each component has a downstream region in reverse complement (RC) orientation to the upstream region of another component, such that α binds *β* and *γ*; *β* binds *γ* and α; and *γ* binds α and *β*. Upon self-assembly, the only possible conformation that can be formed is the one shown in Figure 1a.

Generation of Component Templates. The individual steps undertaken to generate component α are shown in Figure 2; components *β* and *γ* are obtained in a similar manner. The template for component α was obtained by PCR amplification such that a linker restriction enzyme (LRE) site is incorporated in the downstream 5′ end of the fragment. A unique 20 bp sequence was added upstream of all three components to serve as a functional 5′ overhang in the assembled complex. After PCR, both products were digested appropriately and ligated giving the template for one component of the branched DNA. The ligation product was then cloned into a bacterial vector and sequenced to isolate a pure, error-free template. The double stranded component template was amplified by PCR which also incorporated a further restriction site (BRE) and five phospho-

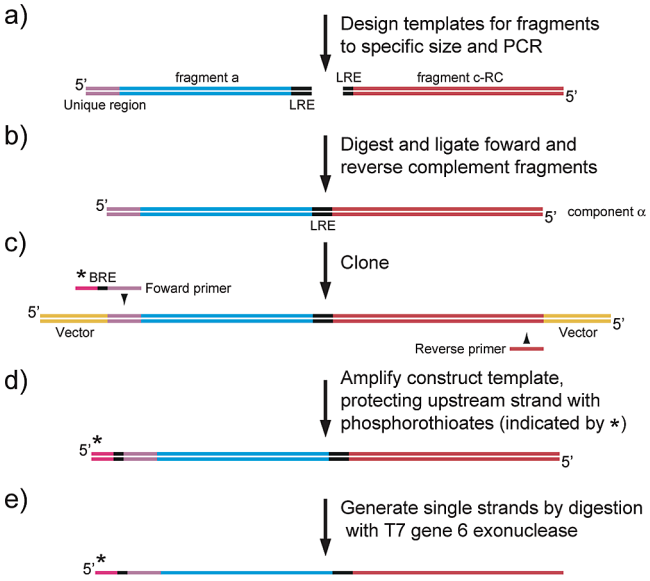

Figure 2. Schematic illustration for the fabrication of component α. (a) PCR primers were designed for the amplification of each fragment (i.e., fragments a and c-RC; for a detailed list of PCR primers and sequences used, see the Supporting Information). (b) Each fragment was digested and the fragments were ligated. (c) Ligated component templates were cloned into pGEM-T easy (Promega). (d) Plasmids were used as templates to amplify the individual components with the forward primer modified with five phosphorothioates on the 5′ end. (e) Double stranded products with one strand protected with phosphorothioates were digested with T7 exonuclease.

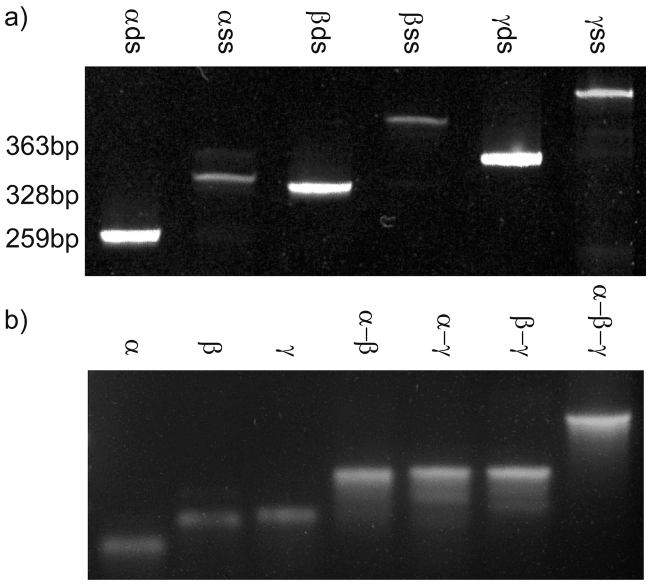

Figure 3. (a) T7 Gene 6 exonuclease digested products of α, β, γ (ds, double stranded undigested controls; ss, single stranded T7 gene 6 treated products). Indicated on the left is the size in base pairs of double stranded DNA. We note that, as expected, single stranded DNA fragments run slower than double stranded DNA fragments. (b) 2% Agarose Gel shift assay of individual components of the three-way complex: [lanes 1−3] individual T7 gene 6 exonuclease digested products; [lanes 4−6] two components of the three-way complex annealed; [lane 7] all three components of the three-way complex annealed.

rothioates on the 5′ end of the upstream strand (Figure 2). The results of this PCR are shown on a 5% polyacrylamide gel in Figure 3a (lane 1; lanes 3 and 5 show the results of the equivalent PCR for components β and γ).

Production of Single Stranded DNA Components. It has been shown that a modification at the 5′ end of double stranded

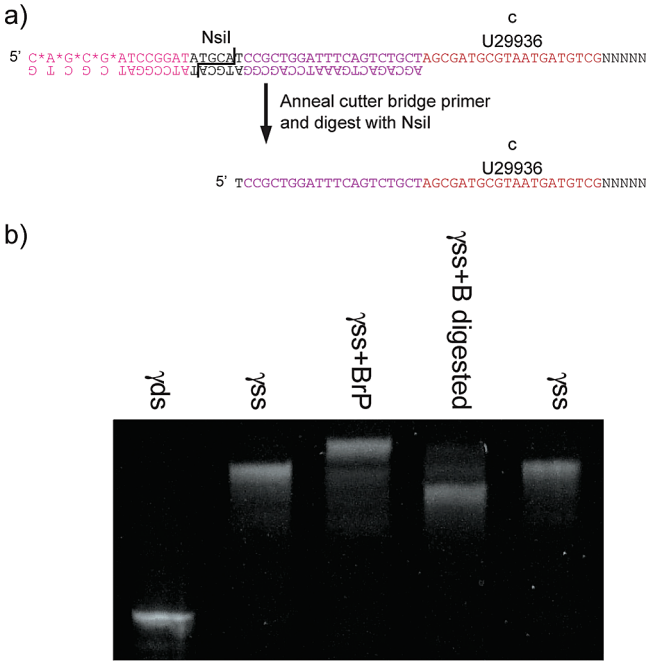

Figure 4. (a) Schematic of method to remove phosphorothioate protection groups from T7 gene 6 exonuclease digested components. A complementary oligonucleotide cutter bridge is used to create a double stranded Nsi I (New England Biolabs) binding site. (b) 20% PAGE gel of digested products showing removal of phosphorothioate blocking region from component γ: [lane 1] γ double stranded (γds); [lane 2] γ single stranded (γss); [lane 3] γ single stranded + bridging primer (BrP); [lane 4] γ single stranded + bridging primer digested with 50 units of Nsi I; [lane 5] γ single stranded.

DNA with five phosphorothioates blocks the 5′ exonuclease activity of T7 gene 6 exonuclease.[24] We exploited this blocking capability and generated the single stranded components from the double stranded PCR product by digestion with T7 exonuclease (Figure 2e). This reaction has been assessed by standard PAGE gel analysis, and the results are shown in Figure 3a. Lanes 1, 3, and 5 show the undigested, double stranded PCR products α, β, and γ, respectively, and lanes 2, 4, and 6 show the respective digested single stranded components. As expected, the single stranded bands appear much fainter in the PAGE gel and run slower. We note that for all components, no remaining double stranded DNA could be detected, strongly indicating that the digestion reaction was very efficient.

Removal of Phosphorothioate Protection Groups from Single Stranded Component Template. Some applications may be hindered by the presence of phosphorothioates, and in these instances, an additional step to remove the phosphorothioates is required, which is demonstrated here. The five phosphorothioates at the 5′ end of the resulting single stranded components are removed using a corresponding bridge primer (BrP) as illustrated for component γ in Figure 4. This bridging oligonucleotide was used to make the 5′ end of the component temporarily double stranded to allow digestion by a restriction enzyme similar to the method used by Peale et al.[25] Figure 4b shows a standard 20% PAGE gel analysis of the digestion reaction demonstrating the successful cutting of the phosphorothioated 5′ end from the single stranded γ component. Lanes 2 and 5 show the complete, i.e., containing the five phosphorothioates at the 5′, single stranded component γ. Lane 3 shows component γ with the appropriate bridging primer hybridized and a clear shift with respect to lanes 2 and 5, owing to the higher molecular weight of this component, is observed. Finally, lane 4 shows component γ with the five phosphorothioates

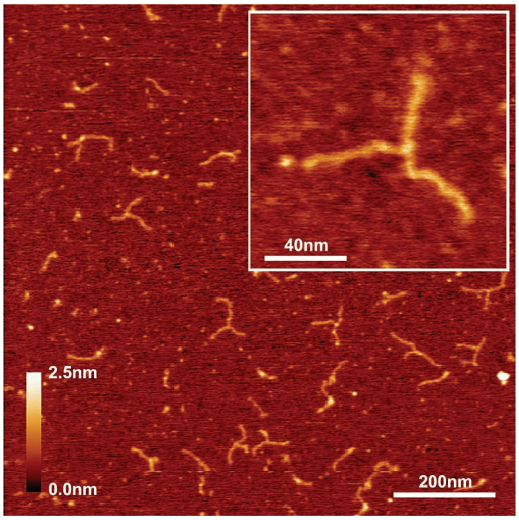

Figure 5. Atomic force micrograph of three-way branched DNA structures on a mica substrate.

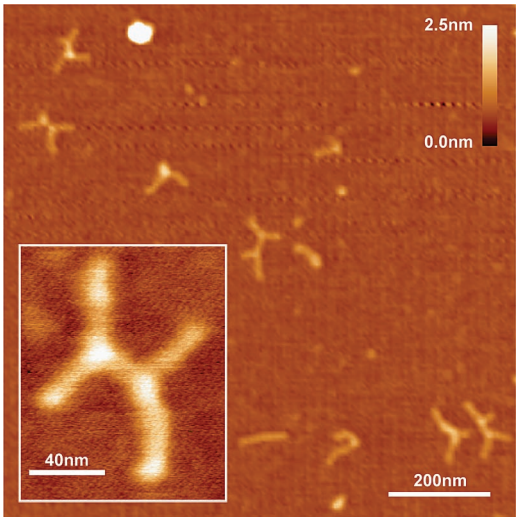

Figure 6. Atomic force micrograph of a branched DNA structure containing two branch points on a mica substrate.

removed, and again, a clear shift with respect to the unmodified component γ (lanes 2 and 5), owing to the reduction in length and thus molecular weight, is observed.

Assembly of Branched DNA Structures. DNA complexes were self-assembled by mixing each component at equimolar concentrations in 1X hybridization buffer consisting of 5 mM HEPES pH 7.0, 2 mM $MgCl_2$, and 0.5 mM EDTA.[26] The component mixture was annealed by heating to 95 °C for 10 min and slowly reducing the temperature in a stepwise fashion designed to approximate the temperature decay of 1.6 L of water in a glass beaker (see the Supporting Information for details). The result of this annealing process was analyzed by gel shift assay on a 2% agarose gel (Figure 3b). Lanes 1, 2, and 3 show the individual components (α, β, γ), respectively, lanes 4, 5, and 6 show DNA complexes with two components annealed ($\alpha-\beta$, $\alpha-\gamma$, $\beta-\gamma$), respectively, and lane 7 shows the complete, assembled three-arm DNA complex ($\alpha-\beta-\gamma$). A substantial increase in apparent size of the product was found as extra components were introduced. We note that there are no visible traces of either individual single stranded components or incompletely assembled complexes when all components were added (lane 7), indicating that the self-assembly process was reasonably efficient and the designed complex was formed.

AFM Analysis of Branched DNA Structures. A more detailed analysis of the DNA complexes was performed by AFM imaging. Figure 5 shows a typical AFM image, and the inset shows a high-resolution scan of one of the three-arm complexes. The measured dimensions (Figure 5, inset) of the three arms, 68, 40, and 53 nm, respectively, correspond well with the expected lengths of the three arms (77, 41, and 53 nm). To verify further the ability of this technique to generate complex branched structures, we extended the single branched three-arm complex to include a second three-way branch point giving rise to a four-arm complex (for details of the design see the Supporting Information). A typical atomic force micrograph of this complex is given in Figure 6.

Conclusion

In conclusion, we have demonstrated an enzymatic technique to generate large branched DNA complexes of defined topologies. In contrast to previously published techniques to generate multi-arm DNA junctions using synthetic DNA oligonucleotides, our technique does not impose the same restrictions on the length of the constituent DNA.

Acknowledgment. This work was in part funded by the EPSRC.

Supporting Information Available. A temperature decay curve, and a detailed list of PCR primers and sequences used. A comprehensive list of oligonucleotide sequences used to generate and ligate the fragments for the single branched DNA complex (components α, β, γ) and the double branched DNA complex (components α, δ, γ, and ϵ). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Seeman, N. C. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 225−248.
(2) Liu, Y.; Lin, C.; Li, H.; Yan, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 4333−4338.
(3) Braun, E.; Eichen, Y.; Sivan, U.; Ben-Yoseph, G. *Nature* **1998**, *391*, 775−778.
(4) Mbindyo, J. K. N.; Reiss, B. D.; Martin, B. R.; Keating, C. D.; Natan, M. J.; Mallouk, T. E. *Adv. Mater.* **2001**, *13*, 249−255.
(5) Harnack, O.; Ford, W. E.; Yasuda, A.; Wessels, J. M. *Nano Lett.* **2002**, *2*, 919−923.
(6) Mertig, M.; Ciacchi, L. C.; Seidel, R.; Pompe, W.; Vita, A. *Nano Lett.* **2002**, *2*, 841−844.
(7) Richter, J.; Seidel, R.; Kirsch, R.; Mertig, M.; Pompe, W.; Plaschke, J.; Schackert, H. K. *Adv. Mater.* **2000**, *12*, 507−511.
(8) Tanaka, K.; Tengeiji, A.; Kato, T.; Toyama, N.; Shionoya, M. *Science* **2003**, *299*, 1212−1213.
(9) Seeman, N. C. *Mater. Today* **2003**, *6*, 24−29.
(10) Welch, J. B.; Duckett, D. R.; Lilley, D. M. J. *Nucleic Acids Res.* **1993**, *21*, 4548−4555.
(11) Zhong, M.; Rashes, M. S.; Kallenbach, N. R. *Biochemistry* **1993**, *32*, 6898−6907.
(12) Zhong, M.; Rashes, M. S.; Marky, L. A.; Kallenbach, N. R. *Biochemistry* **1992**, *31*, 8064−8071.
(13) Mao, C.; Sun, W.; Seeman, N. C. *Nature* **1997**, *386*, 137−138.
(14) Seeman, N. C. *Nanotechnology* **1991**, *2*, 149−159.
(15) Chen, J.; Seeman, N. C. *Nature* **1991**, *350*, 631−633.
(16) Shih, W. M.; Quispe, J. D.; Joyce, G. F. *Nature* **2004**, *427*, 618−621.
(17) Yan, H.; Park, S. H.; Finkelstein, G.; Reif, J. H.; LaBean, T. H. *Science* **2003**, *301*, 1882−1884.
(18) Keren, K.; Krueger, M.; Gilad, R.; Yoseph, G.; Sivan, U.; Braun, E. *Science* **2002**, *297*, 72−76.
(19) Cooper, J. P.; Hagerman, P. J. *J. Mol. Biol.* **1987**, *198*, 711−719.

(20) Cramer, T.; Volta, A.; Blumen, A.; Koslowski, T. *J. Phys. Chem. B* **2004**, *108*, 16586−16592.
(21) Li, Y.; Tseng, Y. D.; Kwon, S. Y.; D'Espaux, L.; Bunch, J. S.; McEuen, P. L.; Luo, D. *Nat. Mater.* **2004**, *3*, 38−42.
(22) Shchepinov, M. S.; Mir, K. U.; Elder, J. K.; Frank-Kamenetskii, M. D.; Southern, E. M. *Nucleic Acids Res.* **1999**, *27*, 3035−3041.
(23) Rasband, W. S. *ImageJ*; National Institute of Health, Bethesda, Maryland, 2004; http://rsb.info.nih.gov/ij/.
(24) Nikiforov, T. T.; Rendle, R. B.; Kotewicz, M. L.; Rogers Y. H. *PCR Methods Appl.* **1994**, *3*, 285−291.
(25) Peale, F. V.; Mason, K.; Hunter, A. W.; Bothwell, M. *Anal. Biochem.* **1998**, *256*, 158−168.
(26) Mao, C. D.; Sun, W. Q.; Seeman, N. C. *J. Am. Chem. Soc.* **1999**, *121* (1), 5437−5443.

BM050890G