NANO LETTERS

2005
Vol. 5, No. 4
719−722

Periodic DNA Nanotemplates Synthesized by Rolling Circle Amplification

Stefan Beyer, Patrick Nickels, and Friedrich C. Simmel*

Department of Physics and Center for Nanoscience, Ludwig-Maximilians-Universität München, Geschwister-Scholl-Platz 1, 80539 München, Germany

Received January 25, 2005; Revised Manuscript Received March 3, 2005

ABSTRACT

Rolling circle amplification (RCA) is an elegant biochemical method by which long single-stranded DNA molecules with a repeating sequence motif can be readily synthesized. In RCA, small circular single-stranded oligonucleotides serve as templates for the polymerization of the complementary strand. A DNA polymerase with an efficient strand displacement activity can copy the circular template without stopping. This results in a long DNA strand with periodic sequence. We here demonstrate that this method, using DNA recognition and biotin–streptavidin binding, provides a simple procedure for DNA-directed nanoscale organization of matter. As an example, a 74 nucleotide (nt) long circular DNA molecule is amplified into a sequence-periodic single strand with a length up to several micrometers. Hybridization of this long periodic DNA template to the biotinylated complement of the sequence motif results in a long DNA duplex with a periodic arrangement of biotin binding sites. On this duplex, streptavidin-coated particles can be organized into one-dimensional arrays. The resulting DNA constructs are characterized by gel electrophoresis and atomic force microscopy.

Biomolecular nanotechnology aims at the construction of complex nanoscale structures using self-assembly and self-organization strategies adopted from biological systems. One of the most prominent examples of biomolecular self-assembly is the molecular recognition between two single strands of DNA with complementary base sequences. These base-pairing interactions have already been used for the construction of a variety of geometric objects[1−4] and also of complex two-dimensional lattices.[5−8]

Only in a few cases these complex DNA nanostructures have also been utilized as scaffolds for the directed assembly of nanoparticles[9] or proteins.[10] There is a considerable body of work on DNA-directed organization of nanoparticles into simpler architectures,[11−17] while other work concerned with functionalization of DNA with nanoparticles[18] or conductive materials[19−22] did not explicitly make use of the base-pairing interactions − even though this is the prime motivation for the usage of DNA in nanoconstruction. Instead, for convenience usually phage DNA (such as λ-DNA) was used. One notable exception is the development of sequence-specific lithography by Keren et al.[23,24] which is based on homologous strand exchange assisted by the recombination protein RecA.

For many applications it would be desirable to produce a long one-dimensional DNA substrate with a designed DNA sequence rather than having to work with a "random" phage genome. Oligonucleotides of arbitrary sequence can be synthesized base-by-base, but the yield of automated synthesis notoriously is very low for sequences much longer than 100 bases. Long DNA duplexes can be produced with some effort using PCR techniques,[25] but without periodic functionalization these DNA duplexes cannot be easily used for the arrangement of nanoobjects. In particular, a periodic duplex is not readily available for hybridization to a target sequence.

We here show how a simple enzymatic technique, rolling circle amplification (RCA), can be used to produce long single strands of DNA which have a repeating sequence with a designed repeat unit on the order of 100 bases. These repeat units can be addressed by hybridization to their complementary DNA sequence. We exemplify the utility of RCA for nanoconstruction by the organization of periodic one-dimensional assemblies of gold nanoparticles.[26]

Rolling circle amplification[27,28] is a variation of the standard DNA polymerization procedure and is used for signal amplification in mutation detection[29−32] and in immunoassays.[33,34] It also occurs naturally, e.g., during replication of viral RNA (cf. ref 35). In RCA, a single-stranded circular DNA (or RNA) substrate serves as the template for a DNA (or RNA) polymerase. The polymerase makes a copy of the circular template, but after completion of the first round it continues to copy the template sequence without stopping.

* Corresponding author. E-mail: simmel@lmu.de; Fax: (+49) 89 2180 3182.

10.1021/nl050155a CCC: $30.25
Published on Web 03/10/2005

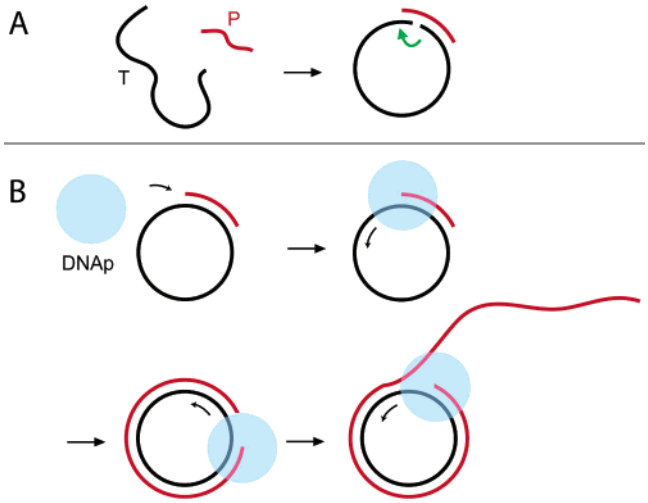

Figure 1. Rolling circle amplification. (A) A circular template is formed by the strands T and P. P holds together the ends of T for ligation and serves as a primer for the polymerization step. (B) A DNA polymerase starts copying the strand T at the primer P. After completion of one round it displaces the newly synthesized strand and starts another polymerization round. Several polymerization rounds lead to the formation of a long single strand with a repeating sequence. The basic repeat unit is the complement of the original template T.

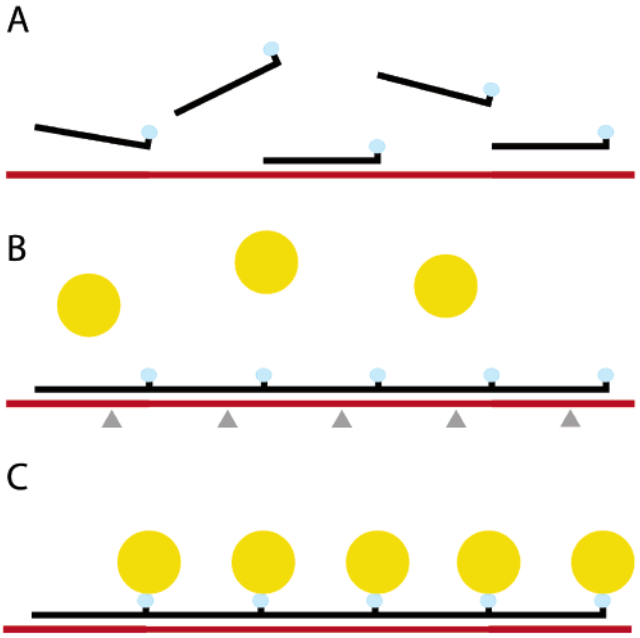

Figure 2. (A) The single-stranded templates (red) can be hybridized to short single strands with the same sequence as the original template T to produce long periodic double-strands. The short strands may be functionalized as indicated, which leads to a periodic arrangement of the functional group. (B and C) As an example, streptavidin-coated gold nanoparticles can be added to an RCA-synthesized template with periodically attached biotin, leading to a one-dimensional organization of the nanoparticles. The gray triangles in B indicate periodically arranged restriction sites that have been incorporated for characterization purposes (see text).

Depending on the reaction conditions and the strand displacement capabilities of the polymerase, this can lead to the synthesis of a long single strand with a repeating sequence that is the complement of the circular template sequence.

The RCA procedure for our particular application is summarized in Figure 1. The circular template is created by hybridizing a 22 base long oligonucleotide P with the 74 nt long strand T. T carries a 5′ phosphate and can be transformed into a circular molecule by T4 ligase (Figure 1 A). The strand P was designed to have one larger (16 bases) and one shorter (6 bases) overlap with T, the latter with a melting temperature higher than room temperature. By this construction, ring formation was favored over linear polymerization of several T strands joined by P strands. After ligation, P acted as a primer for the RCA procedure. The RCA reaction itself was carried out using DNA polymerase from phage ϕ29. This polymerase has exceptional strand displacement properties which is important for an efficient RCA.[29,36] As depicted in Figure 1 B the polymerase starts to synthesize the complement of the template T at the primer P. After one round of polymerization it displaces the newly synthesized strand and continues with polymerization. This ultimately leads to a long strand with a sequence which is the repeated complement of T.

As depicted in Figure 2 A, the resulting single strand with periodic sequence can be transformed into a double strand by hybridizing it to the complement of the repeat unit (or appropriate subunits or multiples of that). If the complementary strands are functionalized, this leads to a periodic one-dimensional arrangement of the functional group.

The result of the RCA reaction was monitored by agarose gel electrophoresis (Figure 3). Gel lane a contains strands T and P after the ligation reaction. Circularization is not complete. The lower band corresponds to unhybridized P, whereas the upper band contains circularized and noncircular T−P. The latter two species are not resolved in this gel. Circularization was checked, however, using lambda exonuclease, which only digests noncircular DNA strands (not shown). Lane b contains the result of the subsequent RCA reaction. The RCA product forms a sharp band with high molecular weight. If either no dNTPs, ϕ29 DNA polymerase, or templates were added to the reaction, no such band was observed. Heat inactivation of the polymerase or skipping the ligation step during template preparation impeded the RCA as well. The length of the resulting single strand was not dependent on the amount of additional primers, dNTPs, or polymerase. Test series with different concentrations of these reactants always resulted in single strands of the same length. This is consistent with earlier findings by other groups. The length of the product can only be varied by stopping the reaction within the first 20 min.[29] Gel lane c contains the result of a hybridization reaction between the RCA product and DNA strands complementary to the repeat unit. A smeared band develops, corresponding to a length of about 10 kbp as judged from a comparison with the 2-Log size standard ladder in lane e. The smearing probably occurs due to imperfect hybridization (not all of the repeat units are hybridized to their complement) and due to cross-linking of several RCA products by the complementary strands. To prove that lane c indeed contains a double-stranded product with a repeating sequence, a restriction site for endonuclease

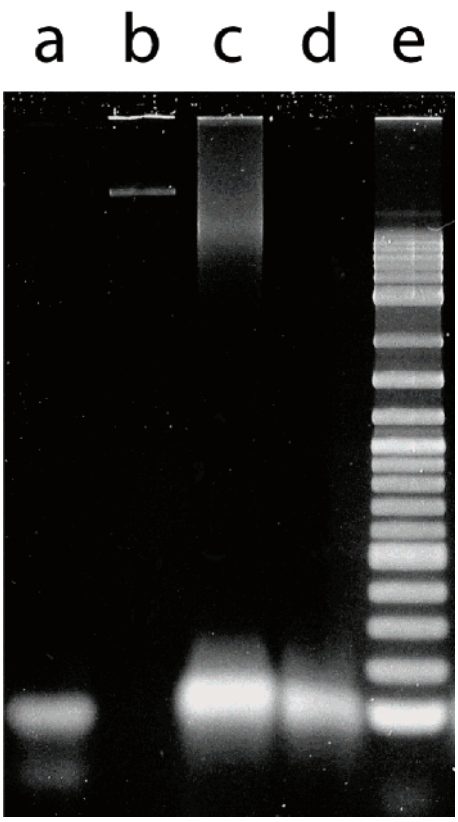

Figure 3. Characterization of the assembly procedure by agarose gel electrophoresis. Lane a contains strands T and P after the ligation procedure. The lower band corresponds to unhybridized P. The upper band contains both ligated and nonligated T−P hybrids, which are not resolved in this gel. Lane b contains the result of the RCA procedure. The amount of circular templates used for the reaction is 1/50 of the amount loaded into lane a. In lane c, the RCA product has been hybridized with an excess of strand T. The hybridization product shows up as a smeared band at around 10 kbp, the unhybridized T is on the bottom of the lane. In lane d, the contents of lane c have been digested with the restriction endonuclease HindIII. The high molecular weight product disappears and the cleavage product shows up at the bottom of the gel. Into lane e, a 2-Log DNA ladder has been loaded for comparison. The lowest band is 100 bp, the highest is 10 kbp long. Exact amounts of material loaded into the gel are given in the Supporting Information.

HindIII was incorporated into the template strands (also indicated in Figure 2 B). Lane d contains the product of a digestion reaction with the contents of lane c. The disappearance of the high molecular weight smeared band indeed proves that lane c contained long double stranded reaction products.

The RCA products were further characterized by atomic force microscopy (AFM). For Figure 4, the RCA product was hybridized to the complementary strand T. The resulting nicks were closed by ligation. The AFM image shows DNA strands with lengths of several hundred nanometers up to a few micrometers, demonstrating the successful generation of long DNA templates with defined sequence from a starting template of only ~25 nm length. For the one-dimensional organization of nanoparticles, the single-stranded periodic DNA template was hybridized to biotinylated DNA strands T-bt with the same sequence as strand T as schematically depicted in Figure 2. Subsequent incubation of the sample

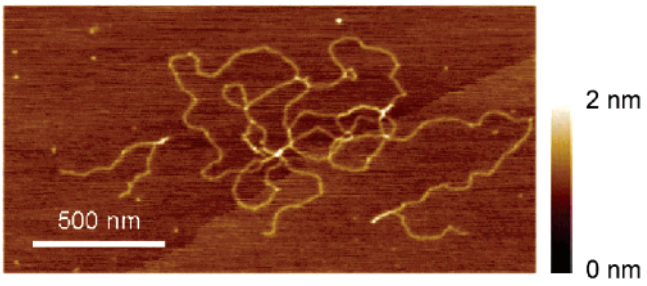

Figure 4. AFM image of the RCA product hybridized to the original template T deposited on mica. The DNA strands have lengths of at least several hundred nanometers. Bright regions (larger height) correspond to several DNA strands sticking together.

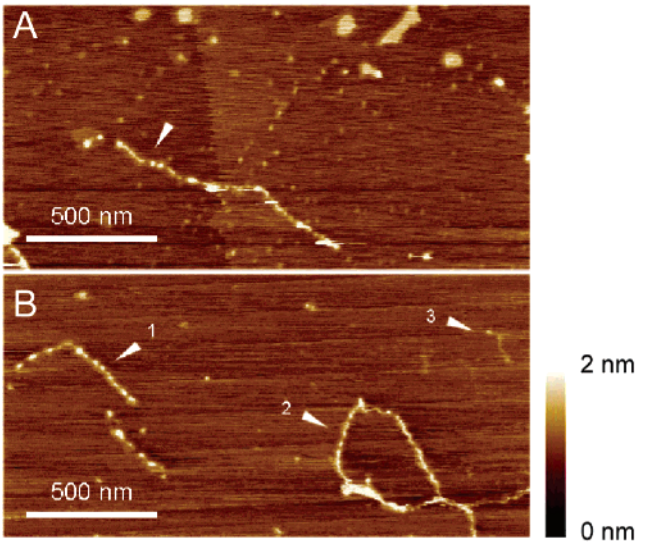

Figure 5. AFM images of the RCA product hybridized to the biotinylated template T-bt and incubated with streptavidin-coated gold nanoparticles with diameter 5 nm. In A, a DNA strand is densely coated with gold nanoparticles with a mutual distance between 30 and 50 nm. In B, densely coated strands can be seen (#1, #2), but also strands which are decorated with only few nanoparticles (#3).

with streptavidin-coated gold nanoparticles with a diameter of 5 nm led to an arrangement of the nanoparticles along the DNA template.[37] In Figure 5, several examples of DNA strands uniformaly covered with gold nanoparticles are displayed. The distances measured between two particles are typically ~30 nm or ~50 nm, which is consistent with nanoparticles binding to every possible binding site with an occasional gap of one single unoccupied biotin. There are other examples where nanoparticle coverage is rather incomplete (Figure 5a, strand #3) and only few particles bind to the template. In general, coverage with nanoparticles is strongly dependent on the total concentration of DNA templates and nanoparticles, the ratio between these concentrations, and the details of the immobilization procedure. High concentrations can lead to the formation of extensively cross-linked networks, as the streptavidin-coated gold particles can link several templates together. In fact, at the concentrations used here, most of the strands with a length comparable to the undecorated strands shown in Figure 4 participate in agglomerated structures. Only shorter strands

as shown in Figure 5 could be imaged isolated from other structures. This problem could be circumvented, however, by using nanoparticles functionalized with a single antibiotin antibody.

In summary, we have shown how an enzymatic method, rolling circle amplification, can be used to produce single-stranded DNA templates with a length of several hundred nanometers which have a repeating sequence with a freely chosen repeat unit of approximately 100 nucleotides. By hybridization to the biotinylated complement of the repeat unit, the templates can be used to periodically arrange streptavidin-coated nanoparticles, or, in principle, any other nanoobject or functional group attached to the appropriate DNA sequence. The resulting 1D structures are, by their very nature, much less complex than the two-dimensional DNA lattices based on multiple crossover molecules or triangular building blocks. As some two-dimensional lattices composed of branched DNA junctions can be constructed from four sets of long periodic DNA strands, e.g., the Winfree tiling of the DAO-E type,[5] it is conceivable that the RCA procedure could even be adapted for the self-assembly of 2D structures. The complexity of one-dimensional structures, however, is already sufficient for many applications and they are relatively simple to produce. One-dimensional chains of nanoparticles can serve as catalysts for subsequent localized materials deposition.[18] Moreover, chains of metal particles with a defined separation are highly interesting in the context of plasmon waveguides.[38] In combination with sequence specific lithography,[23] the periodic templates could be used to produce wires with alternating material composition, e.g., semiconductor islands connected by metal contacts. Finally, an enzyme-based method offers the possibility of in situ synthesis of nanostructures starting from immobilized templates.[32] This could be very helpful for the construction of hybrid systems in which a biomolecular nanostructure has to be embedded into a lithographically defined environment.

Acknowledgment. The authors thank Jörg P. Kotthaus for his continuous support and for hosting the biomolecular nanoscience group in his labs. The authors gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (Emmy Noether program, SI 761/2-1 and -2).

Supporting Information Available: DNA sequences, details on the reaction conditions, AFM imaging procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chen, J. H.; Seeman, N. C. *Nature* **1991**, *350*(6319), 631−633.
(2) Zhang, Y. W.; Seeman, N. C. *J. Am. Chem. Soc.* **1994**, *116*(5), 1661−1669.
(3) Mao, C. D.; Sun, W. Q.; Seeman, N. C. *Nature* **1997**, *386*(6621), 137−138.
(4) Shih, W. M.; Quispe, J. D.; Joyce, G. F. *Nature* **2004**, *427*(6975), 618−621.
(5) Winfree, E.; Liu, F. R.; Wenzler, L. A.; Seeman, N. C. *Nature* **1998**, *394*(6693), 539−544.
(6) Feng, L. P.; Park, S. H.; Reif, J. H.; Yan, H. *Angew. Chem., Int. Ed.* **2003**, *42*(36), 4342−4346.
(7) Yan, H.; LaBean, T. H.; Feng, L. P.; Reif, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*(14), 8103−8108.
(8) Liu, D.; Wang, M. S.; Deng, Z. X.; Walulu, R.; Mao, C. D. *J. Am. Chem. Soc.* **2004**, *126*(8), 2324−2325.
(9) Xiao, S. J.; Liu, F. R.; Rosen, A. E.; Hainfeld, J. F.; Seeman, N. C.; Musier-Forsyth, K.; Kiehl, R. A. *J. Nanopart. Res.* **2002**, *4*(4), 313−317.
(10) Yan, H.; Park, S. H.; Finkelstein, G.; Reif, J. H.; LaBean, T. H. *Science* **2003**, *301*(5641), 1882−1884.
(11) Alivisatos, A. P.; Johnsson, K. P.; Peng, X. G.; Wilson, T. E.; Loweth, C. J.; Bruchez, M. P.; Schultz, P. G. *Nature* **1996**, *382*(6592), 609−611.
(12) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. *Nature* **1996**, *382*(6592), 607−609.
(13) Mucic, R. C.; Storhoff, J. J.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1998**, *120*(48), 12674−12675.
(14) Loweth, C. J.; Caldwell, W. B.; Peng, X. G.; Alivisatos, A. P.; Schultz, P. G. *Angew. Chem., Int. Ed.* **1999**, *38*(12), 1808−1812.
(15) Mitchell, G. P.; Mirkin, C. A.; Letsinger, R. L. *J. Am. Chem. Soc.* **1999**, *121*(35), 8122−8123.
(16) Niemeyer, C. M.; Adler, M.; Gao, S.; Chi, L. F. *Angew. Chem., Int. Ed.* **2000**, *39*(17), 3055−3059.
(17) Weizmann, Y.; Patolsky, F.; Popov, I.; Willner, I. *Nano Lett.* **2004**, *4*(5), 787−792.
(18) Patolsky, F.; Weizmann, Y.; Lioubashevski, O.; Willner, I. *Angew. Chem., Int. Ed.* **2002**, *41*(13), 2323−2327.
(19) Braun, E.; Eichen, Y.; Sivan, U.; Ben-Yoseph, G. *Nature* **1998**, *391*(6669), 775−778.
(20) Richter, J.; Seidel, R.; Kirsch, R.; Mertig, M.; Pompe, W.; Plaschke, J.; Schackert, H. K. *Adv. Mater.* **2000**, *12*(7), 507.
(21) Richter, J.; Mertig, M.; Pompe, W.; Monch, I.; Schackert, H. K. *Appl. Phys. Lett.* **2001**, *78*(4), 536−538.
(22) Nickels, P.; Dittmer, W. U.; Beyer, S.; Kotthaus, J. P.; Simmel, F. C. *Nanotechnol.* **2004**, *15*, 1524−1529.
(23) Keren, K.; Krueger, M.; Gilad, R.; Ben-Yoseph, G.; Sivan, U.; Braun, E. *Science* **2002**, *297*(5578), 72−75.
(24) Keren, K.; Berman, R. S.; Buchstab, E.; Sivan, U.; Braun, E. *Science* **2003**, *302*(5649), 1380−1382.
(25) Rudert, W. A.; Trucco, M., Multimers of specific DNA sequences generated by PCR. In *Genetic Engineering with PCR*; Horton, R. M., Tait, R. C., Eds.; Horizon Scientific Press: Wymondham, 1998; pp. 83−95.
(26) A related approach based on the enzyme telomerase has been taken by Weizmann et al. (ref 17). Using telomerase, however, one is restricted to a specific, and much shorter, repeat sequence (in this case 5′-TTAGGG-3′).
(27) Fire, A.; Xu, S. Q. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*(10), 4641−4645.
(28) Liu, D. Y.; Daubendiek, S. L.; Zillman, M. A.; Ryan, K.; Kool, E. T. *J. Am. Chem. Soc.* **1996**, *118*(7), 1587−1594.
(29) Lizardi, P. M.; Huang, X. H.; Zhu, Z. R.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. *Nature Genetics* **1998**, *19*(3), 225−232.
(30) Baner, J.; Nilsson, M.; Mendel-Hartvig, M.; Landegren, U. *Nucl. Acids Res.* **1998**, *26*(22), 5073−5078.
(31) Dahl, F.; Baner, J.; Gullberg, M.; Mendel-Hartvig, M.; Landegren, U.; Nilsson, M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*(13), 4548−4553.
(32) Chen, Y.; Shortreed, M. R.; Peelen, D.; Lu, M. C.; Smith, L. M. *J. Am. Chem. Soc.* **2004**, *126*(10), 3016−3017.
(33) Schweitzer, B.; Wiltshire, S.; Lambert, J.; O'Malley, S.; Kukanskis, K.; Zhu, Z. R.; Kingsmore, S. F.; Lizardi, P. M.; Ward, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*(18), 10113−10119.
(34) Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W. P.; Wang, M. J.; Fu, Q.; Shu, Q. P.; Laroche, I.; Zhou, Z. M.; Tchernev, V. T.; Christiansen, J.; Velleca, M.; Kingsmore, S. F. *Nature Biotechnol.* **2002**, *20*(4), 359−365.
(35) Branch, A. D.; Robertson, H. D. *Science* **1984**, *223*(4635), 450−455.
(36) Blanco, L.; Bernad, A.; Lazaro, J. M.; Martin, G.; Garmendia, C.; Salas, M. *J. Biol. Chem.* **1989**, *264*(15), 8935−8940.
(37) Niemeyer, C. M.; Ceyhan, B.; Hazarika, P. *Angew. Chem., Int. Ed.* **2003**, *42*(46), 5766−5770.
(38) Maier, S. A.; Kik, P. G.; Atwater, H. A.; Meltzer, S.; Harel, E.; Koel, B. E.; Requicha, A. A. G. *Nature Mater.* **2003**, *2*(4), 229−232.

NL050155A

Beyer et al., Periodic DNA nanotemplates synthesized by rolling circle amplification

Supplementary Information

Chemicals and DNA sequences:

Enzymes and DNA size markers were purchased from New England Biolabs, Beverly, MA, all other chemicals from Sigma Aldrich.

Oligonucleotides were synthesized by biomers.net, Ulm, Germany. Sequences of the oligonucleotides are:

T: 5'-Phosphate-TCGTTTGATGTTCCTAACGTACCAACGCACGGCGAAGCTTT CCGAGGTAGCCTGGAGCATAGAGGCATTGGCTG

T-bt: as T with 5'-biotin

P: 5'-TAGGAACATCAAACGACAGCCA

RCA procedure:

Preparation of circular templates:
A 0.1 µM solution of strands P and T in 20mM TE (Tris-EDTA (ethylene diamine tetraacetic acid), 100mM NaCl, pH 7.8), was heated to 65^{o}C, slowly cooled down to 37^{o}C. T4 ligase and ligase buffer was added (resulting in a dilution by a factor 10) and incubated for 1h at room temperature.

Rolling circle amplification reaction:
Aliquots of 20 µl of ligation reactions were used to template the RCA reactions. The final reaction volume of 100 µl contained 50 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 4 mM dithiothreitol, 200 µg/ml bovine serum albumin, 25 mM dNTP and 10 units of ϕ29 DNA polymerase. The concentrations of P and T were 2 nM, corresponding to the dilution by a factor of 50 compared to the starting solution. The reactions were incubated at 30^{o}C for 3h and heat inactivated at 65^{o}C for 10 min. In order to exchange the buffer, the samples were ethanol precipitated and redissolved in 100 µl 20mM TE buffer, 100mM NaCl, pH 7.8 (product **1**).
For hybridization of the long single-stranded RCA product to the complementary strands T or T-bt, 1.5µl of these strands, both 10µM in TE, 20mM, was added to 10µl of the RCA solution (resulting in product **2**).

Restriction with HinD III
Hind III digestion of the RCA product hybridized to strand T was done using the standard protocol supplied by New England Biolabs (product **3**)

Binding of gold nanoparticles:
The supplier's stock solution of 5 nm Au colloids (Sigma S4188) was diluted 100x in H_2O. 5 µl of that was incubated for 2 h at room temperature with 50 µl of (**2**)

Gel electrophoresis:

Native gel electrophoresis was performed on a 1% agarose gel with 1x TBE (Tris-borate-EDTA, pH 8.3) as gel and running buffer.
Gels were run at 13 V/cm for 1.5 h.
Gels were stained using the gel stain SYBR Gold (Molecular Probes, Oregon) following the manufacturer's protocol.
The amounts of DNA loaded into the gel were adjusted to yield approximately equal brightness in all lanes.
Lane a: 20 µl T+P, 125 nM, Lane b: 10µl of (**1**), Lane c: 12 µl of (**2**), Lane d: 5 µl of (**3**), Lane e: 2-log DNA ladder, 0.1-10 kb

AFM imaging:

Freshly cleaved mica was soaked in 1M $MgCl_2$ for one minute, then dried with N_2. The samples were diluted 100 fold in deionized H_2O and 5µl were applied to the mica for 30 seconds, rinsed with dH_2O and dried with N_2 again. The samples were imaged in tapping mode with a Digital Instruments scanning probe microscope (Dimension 3100) with Nanoscope IIIa controller hardware.