



Characterization of self-assembled DNA concatemers from synthetic oligonucleotides

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ABSTRACT

Studies of DNA–ligand interaction on a single molecule level provide opportunities to understand individual behavior of molecules. Construction of DNA molecules with repetitive copies of the same segments of sequences linked in series could be helpful for enhancing the interaction possibility for sequence-specific binding ligand to DNA. Here we report on the use of synthetic oligonucleotides to self-assemble into duplex DNA concatemeric molecules. Two strands of synthetic oligonucleotides used here were designed with 50-mer in length and the sequences are semi-complementary so to hybridize spontaneously into concatemers of double stranded DNA. In order to optimize the length of the concatemers the oligonucleotides were incubated at different oligomer concentrations, ionic strengths and temperatures for different durations. Increasing the salt concentration to 200 mM NaCl was found to be the major optimizing factor because at this enhanced ionic strength the concatemers formed most quickly and the other parameters had no detectable effect. The size and shape of formed DNA concatemers were studied by gel electrophoresis in agarose, polyacrylamide gels and by AFM. Our results show that linear DNA constructs up to several hundred base pairs were formed and could be separated from a substantial fraction of non-linear constructs.

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1. Introduction

Synthetic oligonucleotides have become an important tool in genomic research, ranging from the assembly of artificial genomes [1] to studying the mechanical properties of individual DNA molecules with optical tweezers [2]. The main reason for using synthetic DNA is that the base sequence can be controlled at will, and one potential application is to study how DNA-binding ligands such as transcription factors recognize the base sequence of DNA. Foot-printing and microarray methods have proven to be powerful tools to quantify the sequence specificity in the binding of peptide mimics [3], but these methods only provide an ensemble-averaged picture. A single-molecule approach to DNA-binding is how the ligand affects the mechanical properties of individual DNA molecules [4–6]. There are two potential effects of ligand binding, changes in the DNA contour length (for instance extension by intercalation) and perturbation of the entropic elasticity of the double helix. Both properties can be measured accurately by mechanical experiments [7] if the single DNA molecule is considerably longer than the persistence length (about 150 bp at 100 mM NaCl).

To date single-molecule investigations of ligand binding have been based on chromosomal DNA with a mixed and complex sequence

composition. We have recently shown that optical tweezers can be sensitive enough to perform single-molecule mechanical experiments on synthetic oligonucleotides, so far with the aim of studying how mechanical tension induces non-native forms in 60 bp duplexes [2]. Another goal is to use oligonucleotides to build synthetic DNA molecules that contain a sequence-designed binding site for a certain ligand but which are about 10 times longer so that changes in the mechanical properties of the native B-form can be measured by optical tweezers. Following the pioneering work of Seeman [8] there has been many efforts to use synthetic oligonucleotides to fabricate DNA-based constructs with intricate structures in both two and three dimensions [9]. Nevertheless, a seemingly simple task of building linear DNA duplexes from oligonucleotides is still of interest to be used as signals amplifier in hybridization-based DNA diagnostics [10,11] and for enhancing cellular uptake [12]. In addition, Filippov and co-workers have presented a careful theoretical analysis of the process of concatemer formation, mainly using thermal melting data to compare with theory but also report a qualitative agreement with data obtained by gel electrophoresis [13].

Here we investigate how two semi-complementary synthetic oligonucleotides 50 bases long can be hybridized into concatemeric trains of duplex DNA molecules that contain repetitive binding sites for Actinomycin D, which was recently studied at the single-molecule level using chromosomal λ -DNA [14]. Our aim is to build such sequence-

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designed DNA molecules which are 500 bp or longer, and combine gel electrophoresis and AFM to investigate the sizes of the concatemers and to which extent the assembly may lead to products which differ topologically from the expected ladder of linear concatemers. To the latter end we rely on our previous report that the circular form of a certain DNA (5486 bp from phage Φ X174) is trapped in native polyacrylamide gels while the linear form of the same DNA size enters the gel [15]. Gel electrophoresis is thus a simple analytical tool to check for the presence of non-linear products in the concatemerization reaction.

2. Material and methods

2.1. Chemicals

Synthetic oligonucleotides AB and A'B' (See Fig. 1a for sequences) were obtained HPLC-purified from ATDBio, Southampton, UK. Stock solutions of oligonucleotides were prepared by dissolving the freeze-dried samples in 5 mM phosphate buffer to a concentration of 25 μ M oligomer. Purity check of the oligonucleotides on denaturing high-resolution (20%) polyacrylamide gels showed that the samples contained a small fraction of oligonucleotides of slightly shorter lengths, the oligonucleotides were used as received. T4 polynucleotide kinase and T4 ligase were obtained from New England Biolabs. DNA stains YO-PRO-1 and SYBR-gold were purchased from Invitrogen and the DNA size markers O'Range Ruler 50 bp DNA ladder (fragments from 50 to 1000 bp) and 500 bp DNA ladder (fragments from 500 to 6000 bp) were from Fermentas. DNA of phage Φ X174 was obtained from Fermentas. All experiments were performed in an aqueous buffer (5 mM phosphate with 0.6 mM KH_2PO_4 and 4.4 mM Na_2HPO_4 at pH 7.6), with addition of NaCl in some cases.

2.2. Design and formation of concatemers

The sequences of the two 50-mer single strand oligonucleotides AB and A'B' (Fig. 1a) were designed to be half-complementary with the intention that they form double-stranded DNA concatemers upon hybridization according to the scheme in Fig. 1b. The concatemers were formed by mixing the AB and A'B' oligonucleotides in equimolar amounts, and then performing an annealing step where the samples were first heated to 90 °C for 5 min after which they were allowed to reach a pre-set temperature (20 °C if not otherwise stated) by passive cooling taking about 10 min. Standard condition for the hybridization was the 5 mM phosphate buffer, but when indicated the ionic strength was increased by adding NaCl.

The intended double-stranded kernel increases in length in steps of 25 base pairs and is flanked at both ends by single stranded parts 25 bases long. Notably, the kernel will contain a nick every 25 bases, but when stated the concatemers were ligated after hybridization. Then the AB and A'B' strands were first incubated with T4 polynucleotide kinase at 37 °C for 30 min in kinase reaction buffer (70 mM Tris-HCl, 10 mM MgCl_2 , 5 mM DTT, pH 7.6), following the produced protocol

but using a 10 fold excess kinase to ensure complete phosphorylation, before the annealing step to secure the 5'-phosphate required by ligase. After annealing T4 DNA ligase was added to the hybridized samples which were incubated for another 30 min in ligase reaction buffer (50 mM Tris-HCl, 10 mM MgCl_2 , 1 mM ATP, 10 mM DTT, pH 7.5) at room temperature. In some case the samples were subjected to repeated annealing and ligation (1–5 cycles). In those cases fresh ligase was added in the last step of each cycle, but kinase was only used at the beginning of the first cycle.

2.3. Gel electrophoresis

Gel electrophoresis was run in TBE buffer (50 mM Tris, 50 mM borate, 1.25 mM EDTA, pH 8.2), using the 50 bp and the 500 bp DNA ladders as overlapping size standards. Before the gel electrophoresis all samples were diluted with incubating phosphate buffer to an equal oligonucleotide concentration of 2.5 μ M and then were loaded on the gel. Non-ligated samples were analyzed in native gels. Native agarose gels (2.5%) were run at 4 V/cm for 3 h at room temperature, and native polyacrylamide gels (5% C; 19:1 monomer/bis) were run at 4 °C and 22.5 V/cm, either at constant field strength for 40 min, or by field inverse gel electrophoresis with electric field running forward time $T_+ = 1$ s, backward time $T_- = 0.1$ s for 50 min, using established protocols [16]. Denaturing electrophoresis was run in polyacrylamide gels containing 7 M urea at 50 °C, 8 V/cm for 30 min. In order to secure DNA denaturation the samples were pre-treated with formamide at 95 °C for 5 min before loading on the gel. The same 50 and 500 bp ladders were used as size markers in the denaturing gels.

To visualize the concatemers and size-markers on the native gels the DNA samples were pre-stained with YO-Pro1. Denaturing gels were post-stained after electrophoresis in a 10,000-fold dilution of the SYBR gold stock solution for 5 min. In all cases the gels were imaged by a Typhoon 9410 scanner using an excitation wavelength of 488 nm and a 520 nm long pass emission filter. The position and fluorescence intensity of the bands on the gels were quantified by the software Image Quant TL.

2.4. Spectroscopic measurements

DNA concentrations were determined by UV absorption spectroscopy at 260 nm. Melting curves were recorded on a Varian Cary 4000 between 20 and 95 °C at a rate of 0.5 °C/min and ramp interval of 0.5 °C.

2.5. AFM-measurements

Mica discs (Agar Scientific) were used as a substrate for AFM-imaging of the DNA. Concatemers assembled under standard conditions were diluted to a DNA concentration of 3 μ M base in a buffer containing 5 mM of HEPES and 2 mM of NiCl_2 . A 20 μ l drop of the diluted DNA solution was applied to the freshly cleaved mica disc and incubated

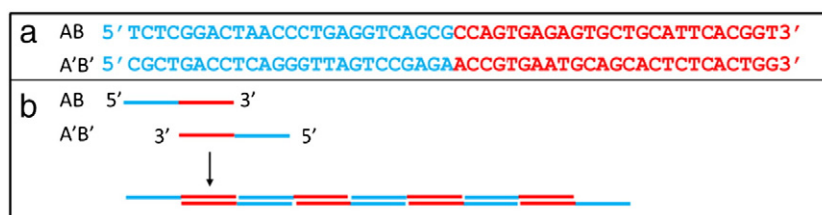


Fig. 1. a) Sequences of the semi-complementary oligonucleotides AB and A'B'. b) The formation of concatemeric DNA duplexes by hybridization of AB and A'B'. The sequence of the 3'-half of the AB-strand (in red) is complementary to the sequence of the 3'-half of the A'B'-strand (also in red). These halves of AB and A'B' can thus form a 25 base pair duplex (red in the bottom part of Fig. 1b). Analogously, the 5'-half of the A'B'-strand (in blue) is complementary to the 5'-half of the AB-strand (also in blue), so they too can form a 25 base pair duplex (blue in bottom part of Fig. 1b).

for 5 min at room temperature. Then the mica sample was rinsed with MilliQ water, before being dried by a weak flux of nitrogen gas. AFM images were collected in air with a NTEGRA Prima, NT-MDT instrument operated in tapping mode using silicon cantilevers (NSG03), and the images were analyzed by the software NT-MDT Nova.

3. Results and discussion

3.1. Formation of concatemers

Gel electrophoresis was used to analyze the products when the oligonucleotides AB and A'B' (Fig. 1) were hybridized. Fig. 2 shows how the size-distribution of the products depends on oligonucleotide concentration, hybridization time, temperature and ionic strength (Fig. 2a–d, respectively). The pattern of discrete and aligned bands under such a wide range of hybridization conditions strongly supports that the oligonucleotides AB and A'B' do form concatemers. Notably our results show a distinct band pattern compared to the electrophoresis results of Filippov et al. [13]. The position of the bands was used to analyze the size of the concatemers, whereas the integrated intensity of each band was used to quantify the relative amount of the different concatemers in the sample.

Fig. 2a shows the results when the hybridization was conducted at different oligonucleotide concentrations (2.5, 5, 10 and 20 μM single strands). It is seen that increasing the oligonucleotide concentration leads to longer concatemers. At 2.5 μM the dominating species is the dimer (ABB'A') and the longest concatemer is about 300 bp, whereas at 20 μM strands the constructs are as long as 1000 bp with a maximum at approximately 350 bp. Fig. 2b shows that increasing hybridization temperature helps forming long concatemers at 37 $^{\circ}\text{C}$; however, concatemers incubated at 60 $^{\circ}\text{C}$ end up with lengths as short as those incubated at 20 $^{\circ}\text{C}$. Melting curves (Fig. 2e) show that concatemers melt between 37 and 58 $^{\circ}\text{C}$. This result explains the result in Fig. 2b lane VIII where concatemers incubated above this melting temperature became melted single strand and end up to short concatemers. Fig. 2c shows that increasing the hybridization time at 20 $^{\circ}\text{C}$ has a negligible effect on the size distribution beyond 2 h. Finally we investigated the effect of added salt, with the aim of reducing the repulsion between the single strands during the hybridization. Fig. 2d shows the size distribution in the presence of increasing NaCl concentrations (at 2.5 μM oligos and 20 $^{\circ}\text{C}$). Starting with no added salt (i.e. only the 5 mM background phosphate buffer) the results show that increasing the salt concentration (25, 50, 100, 200, 300 and 400 mM NaCl) gives longer concatemers in general, but with little enhancing effect beyond 100 mM NaCl.

Our main goal is to obtain long concatemers in an efficient manner. Comparison of the results in Fig. 2d (lane 4) with those in Fig. 2a (lane IV), 2b (lane VII) and 2c (lane XII) indicates that concatemers in the range 100 bp–1000 bp can be obtained with 2.5 μM oligo in 2 h at room temperature by increasing the salt concentration to 200 mM NaCl, thus avoiding the high oligonucleotide concentration, incubation temperature and long incubation times required at low salt. In order to confirm this strategy we reinvestigated the effect of oligo concentration, hybridization temperature and hybridization time at 200 mM added NaCl. The results (Fig. 3) show that in the presence of 200 mM NaCl similarly long concatemers were formed regardless of variations in the other hybridization conditions. This observation supports that adding salt is advantageous to form long concatemers efficiently, so the rest of the experiments were performed in the presence of 200 mM NaCl in addition to the background 5 mM phosphate buffer.

3.2. Concatemer sizes

Fig. 4 compares the apparent sizes of the concatemers with the calibration curve (solid line in Fig. 4) which was obtained by plotting the size of the standards (Fig. 2a) versus the inverse of their mobility. The mobility data of the concatemer products (Fig. 2) were plotted in two ways, differing in whether the single-stranded parts at the ends (Fig. 1) were taken into account or not. The dots in Fig. 4 show the mobility data when the concatemers are assumed to contain only the double stranded part (i.e. 25 bp per monomer). The agreement with the calibration curve is good enough to confirm quantitatively that the concatemers are formed. However, a slight deviation is seen with the shorter concatemers, for two possible reasons. One is the influence of the 25 base single strands at both ends (“tails”), the other is the nicks that the concatemers contain in the absence of ligation. The first possibility was tested by including the tails as an effective additional 25 bp (squares in Fig. 4), which indeed improved the agreement with the calibration curve. Assuming that the two 25 base ss-DNA tails are electrophoretically equivalent to a 25 base pair helix is clearly an approximation but seems reasonable because the correction becomes less significant as the length of concatemers increases, as expected if the deviation is due to an end-effect. By contrast, the possible effect of nicks on the electrophoretic mobility should be insensitive to concatemer length since the nicks occur at 25 bp intervals throughout the assembled DNA molecules. Below we use denaturing gel electrophoresis to investigate the ligation efficiency in the concatemers.

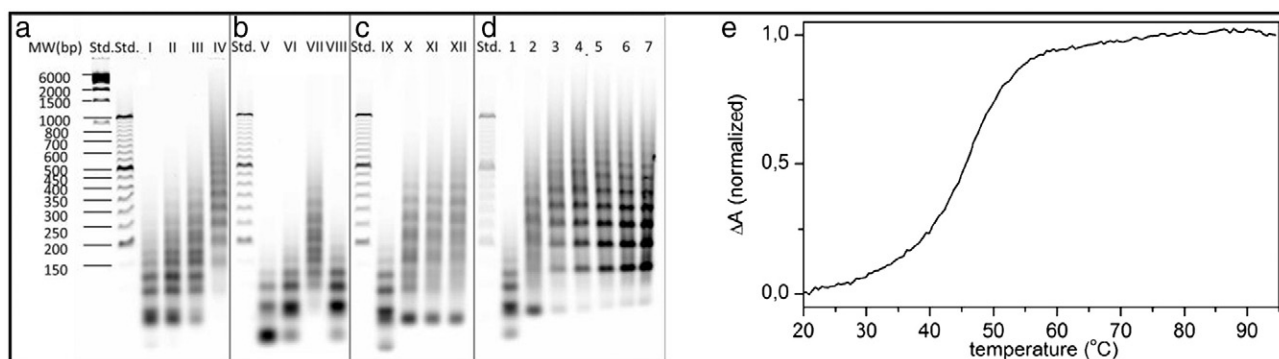


Fig. 2. Electrophoretic gel analysis of the DNA concatemers assembled under various conditions and melting curves for oligonucleotides and concatemers. (a) Oligo concentrations: 2.5, 5, 10 and 20 μM strands (lanes I–IV), with all samples incubated at 20 $^{\circ}\text{C}$ for 2 h before the electrophoresis. (b) Incubation temperature: 4, 20, 37 and 60 $^{\circ}\text{C}$ (lanes V–VIII), at 2.5 μM oligo and 2 h. (c) Incubation times: 2, 24, 48 and 72 h (lanes IX–XII), at 2.5 μM oligo and 20 $^{\circ}\text{C}$. (d) Added salt: 0, 25, 50, 100, 200, 300 and 400 mM NaCl (lanes 1–7), at 2.5 μM oligo, 20 $^{\circ}\text{C}$ and 2 h. During concatemer formation all samples contained a background phosphate buffer (5 mM, pH 7.6); only in panel (d) was the ionic strength higher. Electrophoresis in 2.5% agarose gels at 4 V/cm. In panel (a) two overlapping size standards (Std) are indicated by the size of the fragments in base pairs. The gels in panels (b)–(d) only contained the shorter size standard. (e) UV melting curves of 1 μM 1:1 mixed AB and A'B'.

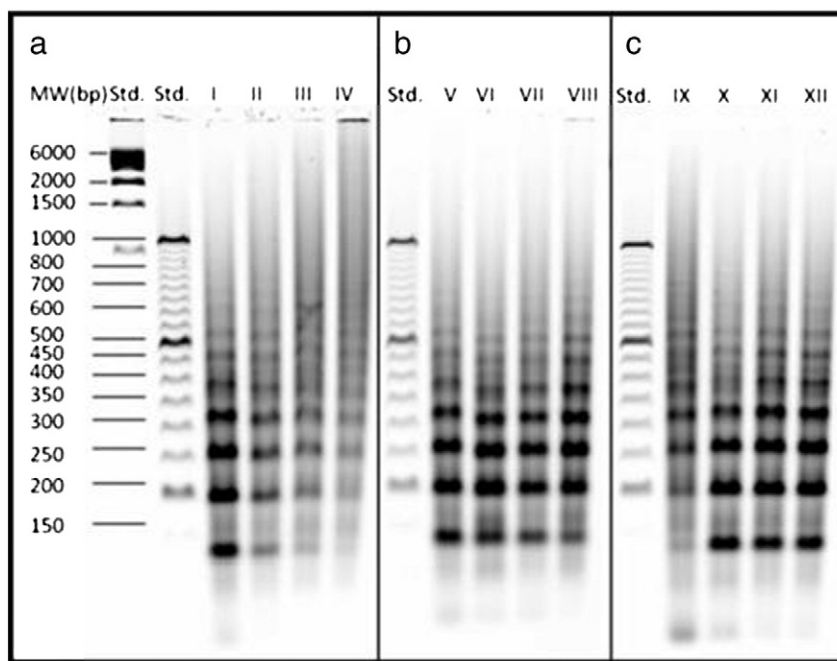


Fig. 3. Electrophoretic analysis of the DNA concatemers formed in the presence of added 200 mM NaCl, under the following conditions. (a) Oligo concentrations: 2.5, 5, 10, and 20 μM (lanes I–IV). (b) Incubation temperatures: 4, 20, 37 and 60 $^{\circ}\text{C}$ (lanes V–VIII). (c) Incubation times: 2, 24, 48 and 72 h (lanes IX–XII). Electrophoresis in 2.5% agarose gels at 4 V/cm.

3.3. Ligation of the concatemers

A ligation step was added to the hybridization protocol with the aim to seal the nicks on the backbones of the concatemers (see Methods). Denaturing gel electrophoresis (Fig. 5a–c) was used to investigate the covalent length of each strand in the concatemers after the ligation. Fig. 5a shows the effect of the added ligase. Without ligase (lane I) the sample only contains the individual AB and A'B' oligos, as expected under the denaturing conditions. In the presence of ligase (lane II) longer strands survive even upon denaturation, showing that the several AB-oligos (and A'B'-oligos in the opposite strand) have been covalently ligated into longer single-strands. Comparison with the size standards under the same denaturing conditions shows that the ligated strands increase in length by the expected 50 bases.

The longest constructs seen in the native agarose gels (Fig. 2) were not observed on the denaturing gels (Fig. 5a), indicating that not all nicks in the concatemers were ligated. To investigate this hypothesis,

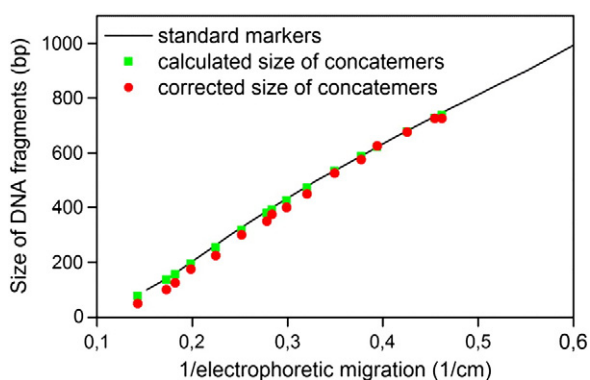


Fig. 4. Plot of the effective DNA size (base pairs) versus the inverse of the electrophoretic mobility. Data points are from Fig. 2d. Effective size of the concatemers excludes (dots) or includes (squares) the 25 base single strand tails at each end (see text for details). Solid curve is calibration curve based on the DNA size markers in Fig. 2a.

the mixed AB and A'B' samples were exposed to between 1 and 5 cycles of heating, cooling and ligation (HCL), either with excess (Fig. 5b) or stoichiometric amounts (Fig. 5c) of the enzymes (kinase and ligase). Comparing Fig. 4b and c shows that excess of enzymes gives longer concatemers in general, but at the obvious cost of more enzymes being spent. Fig. 5c indicates that the amount of long concatemers increases after each cycle while the amount of monomer and dimers decreases correspondingly. This observation is confirmed in Fig. 6 where the results in Fig. 5c are quantified in terms of the amount of concatemers of different sizes after each HCL cycle. It is seen that the amount of short concatemers is decreased by HCL-cycling while longer concatemers are formed to a greater extent. By contrast, the result in Fig. 5b suggests that cycling does not have the same improving effect when the enzymes are in excess. Taken together the results in Fig. 5b, c and Fig. 6 show that the yield of long concatemers at low amounts/efficiency of the ligating enzymes can be improved by HCL-cycling.

The higher size resolution of polyacrylamide gels (Fig. 5) compared to agarose (Fig. 2) reveals that some constructs migrate at positions which do not fit the pattern of 50 base multiples. One example is the weak band which is slightly faster than the strong component at 200 bases in lane II in Fig. 5a, which notably survives the HCL-cycling in Fig. 5b. Such a deviating migration behavior is not likely due to secondary structures in the single strands because of the strongly denaturing conditions, but could possibly be caused by the samples AB and A'B' containing impurities of oligonucleotides which are not of full length (see Materials and methods).

3.4. Tertiary structure of the concatemers

Native polyacrylamide gel electrophoresis was used to analyze the tertiary structure of the hybridization products, because in such gels circular DNA molecules tend to become trapped whereas linear forms with the same numbers of base pairs enter the gel [15]. Fig. 5d shows the results when a hybridized sample of AB and A'B' was analyzed on a native acrylamide gel with a constant electric field of 22.5 V/cm. Fig. 5d lane I shows that a large fraction of the hybridized sample is

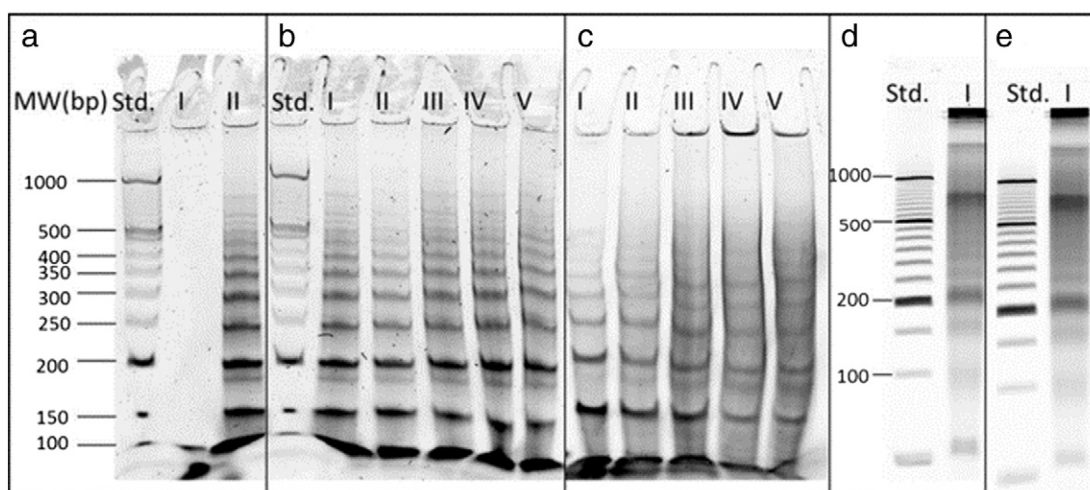


Fig. 5. Polyacrylamide gel electrophoresis (PAGE) of the concatemers. Gels in panels (a)–(c) are denaturing PAGE (7 M urea; 50 °C), gels in panels (d) and (e) are native PAGE. (a) Non-ligated (I) and ligated (II) concatemers. (b) and (c): Oligonucleotides underwent one to five heating, cooling and ligation cycles (denoted I–V), either with excess of ligase and kinase (b) or with a stoichiometric amount of enzymes (c). (d) and (e): native 5% PAGE at 22.5 V/cm either in constant field (d) or in pulsed inversion field electrophoresis (e). Samples in (d) and (e) are the same samples used in (a).

trapped in the well, especially when compared to the size standards (denoted Std in Fig. 5d) designed to contain only linear molecules, an observation which suggests that the hybridization products contain non-linear molecules. The products that do enter the polyacrylamide gel are most likely linear [15] but form less distinct bands than in the agarose gels (Fig. 2) because they are too long to be suitably analyzed in the small pores of polyacrylamide gels.

Studies based on λ -DNA [17,18] have shown that when DNA molecules with complementary ends are hybridized there is a competition between circular and linear concatemer products. The synthetic oligonucleotides used here (Fig. 1) have similar complementary ends so circles are clearly a possible outcome. We used pulsed field electrophoresis to look for circular products because the circles which are trapped in polyacrylamide gels can be released by field-inversion gel electrophoresis (FIGE) so they enter the gel [15]. Fig. 5e (lane I) shows the result when the sample in Fig. 5d (lane I) was analyzed by FIGE in the same native polyacrylamide gel. It is seen that a large fraction of the sample still remains trapped in the well, suggesting that the nonlinear hybridization products are not simply circles.

The possibility of more complicated hybridization products than circles was investigated through direct imaging by AFM, and the images in

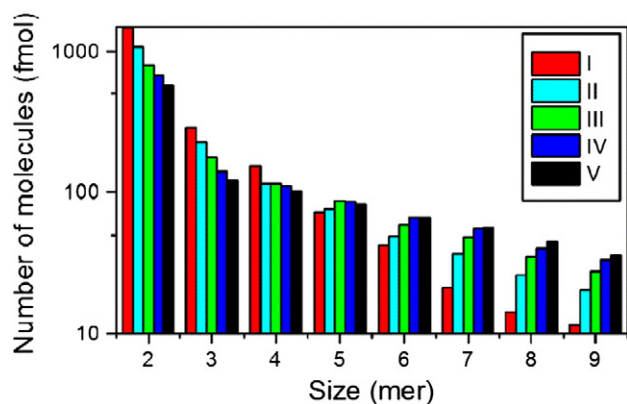


Fig. 6. Quantification of the amount of concatemers in the cycling experiments (data from Fig. 5c). The number of molecules in each of the concatemers is presented for samples that underwent one to five cycles of heating and ligation (indicated by I to V).

Fig. 7 support that the products exhibit a wide range of size and shape. Fig. 7a shows well-spread objects which most likely are DNA molecules because the height of about 0.5 nm (Fig. 7d) agrees with previous AFM studies of DNA in air [19]. The lengths of the constructs vary between 20 and 500 nm which are consistent with the sizes of the concatemers (50–1500 bp) observed in agarose gel electrophoresis (Fig. 2). Fig. 7b and c shows that in addition there are considerably larger and entangled constructs, which may explain why some products are trapped in the wells even during pulsed field electrophoresis (Fig. 5e).

3.5. Purification of linear DNA concatemers

For future studies of DNA-ligand binding on single molecule level, linear concatemers have to be separated from the other mixture shaped products. Gel electrophoresis result has shown that only linear concatemers could migrate into native polyacrylamide gel at constant field (Fig. 5d). Therefore, to obtain linear concatemers with uniform length, hybridized AB and A'B' sample was first running in native PAGE to separate linear concatemers out of non-linear structure (Fig. 8 left). Then bands at desired length (in this experiment 200 bp and 700 bp) were cut off and soaked in 1× TBE buffer at 40 °C for 36 h. Collected sample solutions were treated with freeze drying and checked by native PAGE again to confirm the purification (Fig. 8 right). Result shows that clear bands corresponding to aimed length with 200 bp and 700 bp are displaying at desired positions, which demonstrates that this purification method is easy and feasible.

4. Conclusions

The main purpose of the present study is to assemble synthetic oligonucleotides into sequence-designed DNA molecules which are long enough (about 500 bp) to study ligand binding to B-form DNA using optical tweezers. The results show that hybridization in bulk solution can be used to form concatemers which are long enough (Fig. 2), although the results of gel electrophoresis (Fig. 4d,e) and AFM-imaging (Fig. 7) indicate that a substantial fraction of the products are not the linear concatemers needed for the single-molecule experiments. If the complementary ends are responsible for the undesired non-linear products one possible resolution is to build the synthetic concatemers while one of the ends is attached to a solid surface and in effect inactivating it. In

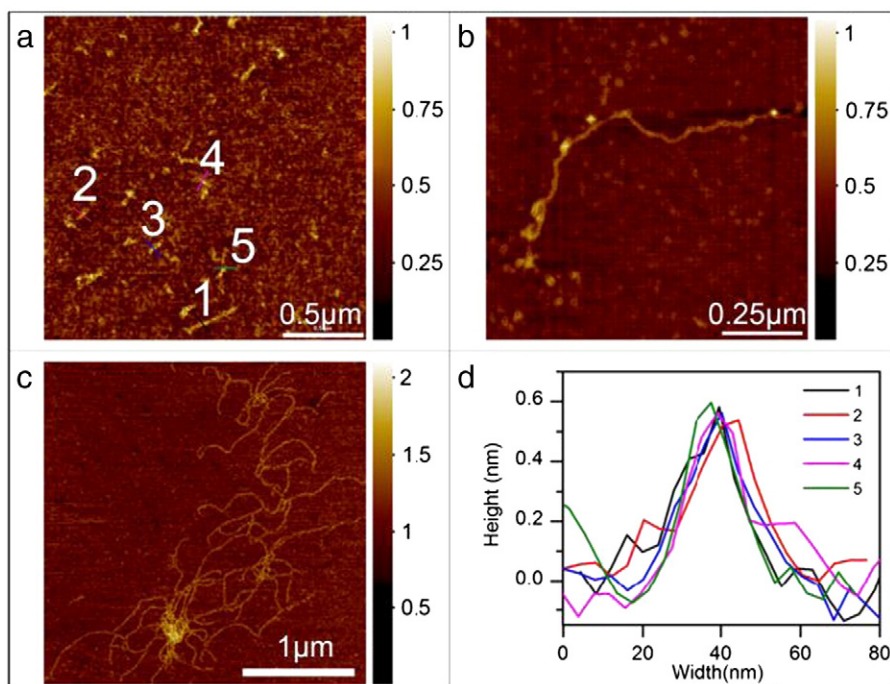


Fig. 7. AFM images of the hybridization products. Panels a), b) and c) images in air showing the variation in the tertiary structure of the hybridization products. a) Short constructs; b) longer and slightly entangled products; c) large and highly entangled products. d) Typical height profiles of 5 individual constructs. The location of the profiles is displaying in panel a).

fact, the 5–7 kbp “cassette” intermediates used in the construction of the synthetic *Mycoplasma genitalium* genome were made by assembling different synthetic oligonucleotides using a solid phase. At any rate the linear constructs can be isolated by gel electrophoresis (Fig. 2), and the femto-molar amounts of gel-separated products (Fig. 6) are high enough for single-molecule experiments according to our previous study of synthetic DNA [2].

The results also shine some light on the mechanism by which the concatemers are formed. The observation that cycling the heating–cooling–ligation process increases the lengths of the concatemers (Fig. 6) indicates that the hybridization process is under kinetic (rather than thermodynamic) control, as also supported by our observation (Fig. 2) that an increased ionic strength speeds up the concatemer formation [17]. However, we cannot rule out the possibility that the cycling

only breaks up concatemers that contain oligonucleotides which cannot be ligated (for instance because they lack a 5′-phosphate), and so gives a fresh start to a successful ligation. It should also be noted that a kinetic control of the concatemerization could be the result of formation of slowly resolving secondary structures in the individual oligonucleotide, a question which is outside the scope of the present investigation to resolve.

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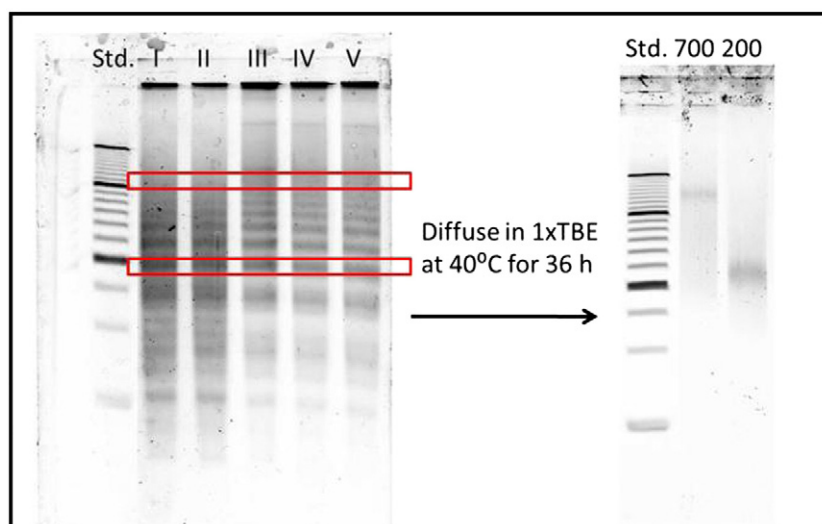


Fig. 8. Purification of linear concatemers in native PAGE. (left) Separation of linear concatemers from hybridized sample, (right).

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