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The construction of DNA molecules of figure-eight structure

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Abstract

Using DNA molecules to construct a structural scaffold for nanotechnology is largely accepted. In this article, we report on two methods for constructing a figure-eight structure of DNA molecules having a relatively high yield that could be used further as a scaffold for nanotechnology applications. In the first method, two plasmids were constructed that, on digestion with a restriction endonuclease producing nicks in the corresponding sites and after heating, produced complementary single-stranded sequences, enabling the plasmids to hybridize to each other and forming a figure-eight structure. The formation of the figure-eight structure was analyzed by restriction analysis and gel electrophoresis as well as by atomic force microscopy. The second method makes use of the bacteriophage M13 that is obtained as either a single- or double-stranded circular DNA molecule. Two M13 molecules harboring complementary sequences were constructed and produced a figure-eight structure on hybridization. The methods described here could be used further for the construction of nanoelectronic devices

Keywords: Figure-eight DNA structure; Plasmids; M13 vectors

Bioelectronics and nanoelectronics are believed to hold the future for nanotechnology. Controlling the structure and properties of two- and three-dimensional assemblies of molecules is the essence of supramolecular chemistry as well as of materials chemistry and molecule-based electronics [1,2]. In addition to their major role as the carriers of genetic information, nucleic acids are a versatile material for designing nanometer-scale structures. Following Seeman's pioneering work in using DNA as building blocks for constructing ordered and well-defined structures, different groups have demonstrated the use of DNA synthons as assembly units for the construction of functionalized molecular networks bearing electrically active components [3,4]. The inherent versatility of organic chemistry and the potential application of π-conjugated organic materials in structuring molecular electronic components have focused enormous efforts in the preparation and characterization of new organic-based electronics components. In parallel, efforts are being made to harness biological processes and molecules as building blocks and assemblers of nanocomponents. DNA-programmed assembly of nanoelectronic devices in a reasonable quantity and the reduction of errors in the self-assembly process are perhaps the major challenges delaying even more rapid progress in this field [5–11].

In our studies on the production of nanoelectronic devices, we faced the need for constructing a figure-eight structure composed of DNA. We report here on two methods for producing a relatively large quantity of this structure that could be further used for building bioelectronic devices.

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Materials and methods

Construction of plasmids

The pairs of oligonucleotides Sk-1.A and Sk-1.B, as well as Sk-2.A and Sk-2.B (100 μM each), were annealed to each other (Fig. 1) and cloned into *Pst*I/*Sal*I digested pBluescriptII SK(+) (Stratagene) to produce the plasmids Sk-in-1 and Sk-in-2 (Fig. 1). Large quantities of plasmid DNA were obtained by the transformation of Xl1-blue *Escherichia coli* cells and plasmid preparation (Wizard plasmid preparation, Promega).

Formation of figure-eight structure of two plasmids

First, 1 μg of the plasmids Sk-in-1 and Sk-in-2 was nicked with 10 U of N.BbvC IA (New England Biolabs) at 37 °C for 1 h. A mixture of complementary oligonucleotide (Fig. 2) and the nicked plasmid (10:1 molar ratio) was heated to 70 °C for 10 min and cooled down slowly to 25 °C. The annealed oligo was removed by using Centricon 100 (Amicon). The two plasmids were then mixed together at a concentration of 1.25 μM in H_2O and incubated at 37 °C for 16 h.

Production of large quantities of single-stranded M13mp18 and M13mp19 molecules

Xl1-blue cells were transformed with the corresponding plasmid [12] and grown overnight at 37 °C with vigorous stirring. The bacteria cells were pelleted by centrifugation at 10,000 rpm for 15 min, and a solution

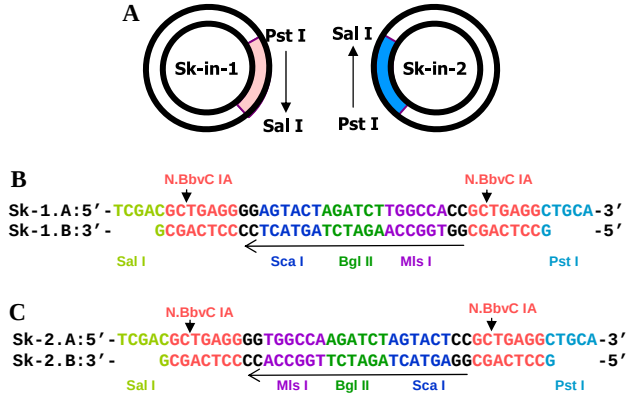

Fig. 1. (A) Plasmids for formation of the figure-eight structure. (B) Oligonucleotides Sk-1.A and Sk-1.B were annealed and cloned into the pBluescript Sk plasmid digested with restriction enzymes *Pst*I and *Sal*I, producing the plasmid Sk-in-1. (C) The Sk-in-2 plasmid was constructed in the same way using the annealed oligonucleotides Sk-2.A and Sk-2.B. The site of the nick formation enzyme N.BbvC IA (two sites per plasmid) and the restriction endonuclease sites used to analyze the formation of the double-stranded DNA in the annealed plasmids are indicated in color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

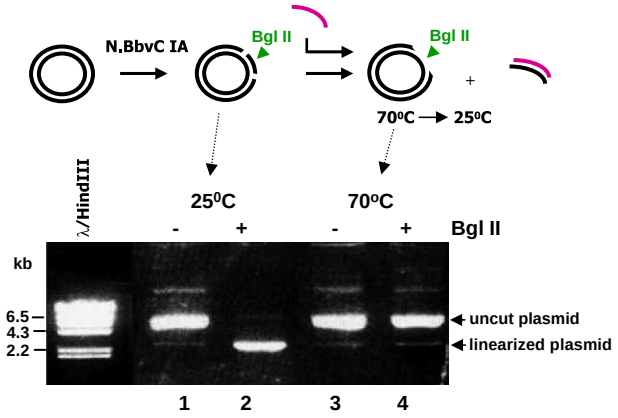

Fig. 2. Removal of the single-stranded DNA segment produced by N.BbvC IA digestion. The plasmids were digested with the N.BbvC IA enzyme, followed by heating to 70 °C, adding an excess of oligonucleotide complementary to the single-stranded DNA segment removed from the plasmid, and annealing by slow cool down, as shown schematically. The plasmid is shown before and after heating for the release of the DNA segment (25 and 70 °C, respectively). The *Bgl*II site is located such that the removal of the single-stranded DNA segment leaves a single-stranded DNA that would not be cleaved by this enzyme (lanes 1 and 2). However, before removing the segment by heating, the nicked plasmid is cleaved and linearized by *Bgl*II (lane 2).

containing 2.5 M NaCl and 20% polyethylene glycol (PEG,[1] 8000 MW) was added to the supernatant and stirred at room temperature for 1 h [13]. The bacteriophages were collected by centrifugation at 10,000 rpm for 20 min at 4 °C. The bacteriophage pellet was then dissolved in 10 ml of 10 mM Tris–Cl (pH 8.0). An equal volume of equilibrated phenol was added and mixed by vortexing vigorously for 2 min, followed by centrifugation for 5 min at 5000 rpm (Sorvall SS-34 rotor). The aqueous phase was collected and extracted again with 10 ml of phenol/chloroform. The single-stranded plasmid was then collected by ethanol precipitation and resuspended in buffer TE (10 mM Tris–Cl and 1 mM ethylenediamine tetraacetic acid [EDTA], pH 8.0).

Analysis of single-stranded DNA and formation of figure-eight structure of the two plasmids

For analysis, 100 ng was digested with 10 U of mung bean exonuclease (New England Biolabs) for 30 min at 37 °C. Alternatively, 250 ng of the same DNA was digested with 5 U of *Hin*dIII and *Bgl*II restriction enzymes (Fermentas). To form the figure-eight structure, 150 ng of each molecule were mixed together in 6 μl of H_2O and incubated at 50 or 60 °C for three separate time intervals (15, 30, and 45 min).

[1] *Abbreviations used:* PEG, polyethylene glycol; EDTA, ethylenediamine tetraacetic acid; AFM, atomic force microscopy.

Atomic force microscope imaging

For the plasmid dsDNA constructs, a 20 µl solution containing 2.5 ng (10 nM) of the figure-eight structure in buffer I (4 mM Hepes [pH 7.4], 10 mM NaCl, and 2 mM $MgCl_2$) was dropped onto freshly cleaved mica, which was washed with H_2O after standing for 4 min. The sample was dried under a stream of argon.

For the analysis of the ssDNA M13 constructs, a solution of 2 ppm poly-L-lysine in water was made by diluting a 0.1% aqueous solution of poly-L-lysine hydrobromide (Sigma). The 2 ppm solution (30 µl) was deposited onto freshly cleaved mica for 5 min, followed by rinsing the surface with water and drying under a stream of nitrogen. A 5 µl droplet of the figure-eight structure (10 ng/µl of DNA) in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) was deposited on the treated mica for 1 min and then rinsed with water and dried under a stream of nitrogen [14,15].

Observations were performed using an atomic force microscope (Dimension 3100, Digital Instruments) in the tapping mode in air with RTESP (silicon) cantilevers (Nanoprobe). Fields of less than 10 µm were scanned at 1 Hz per scan line.

Results and discussion

Construction of plasmids to form figure-eight structure

To construct the figure-eight structure by combining two double-stranded DNA plasmids, we designed two pairs of oligonucleotides. Annealing each pair formed a double-stranded DNA molecule with cohesive ends for the restriction sites of enzymes *Sal*I and *Pst*I (Fig. 1). Two sites for the nick-forming enzyme N.BbvC IA were introduced into each double-stranded molecule, as shown in Fig. 1B. The N.BbvC IA sites were separated by 22 nt of double-stranded DNA harboring three restriction sites for enzymes *Mls*I, *Bgl*II, and *Sca*I (Fig. 1B). Because there is no *Bgl*II site in the SK plasmid, introducing this site into the DNA fragment allowed us to analyze easily whether or not this sequence is in the form of single- or double-stranded DNA. Each pair of oligonucleotides was annealed and then cloned into *Pst*I/*Sal*I digested pBluescript II SK(+) plasmid to form the two plasmids Sk-in-1 and Sk-in-2 (Fig. 1A).

The next step in the construction of the figure-eight structure was to remove the 22 nt segment between the N.BbvC IA sites. To this end, the two plasmids were incubated with this enzyme, introducing nicks at the sites indicated in Fig. 1A. The 22-single-stranded DNA segment was removed from the plasmid by heating to 70 °C (Fig. 2). To prevent annealing of this segment back to the plasmid, an excess amount of oligonucleotide complementary to the removed segment was added and the annealed double-stranded DNA fragment was removed by centrifugation in Centricon 100 (Fig. 2). The efficiency of the formation of the single-stranded segment step was analyzed by digestion with the restriction endonuclease *Bgl*II, which cut at the middle of the segment to be removed (Figs. 1 and 2). As expected, the plasmid was linearized following treatment by the nicking enzyme N.BbvC IA but prior to the removal of the segment by heating (Fig. 2). However, no linearization was observed following heating to 70 °C, indicating that the *Bgl*II site became single-stranded as a result of the removal of the single-stranded DNA segment (Fig. 2). The absence of any linearized plasmid at this stage indicated that the formation of the nicked plasmid and consequential removal of the single-stranded DNA is very efficient. This process was performed on both plasmids Sk-in-1 and Sk-in-2.

Formation of figure-eight structure

The two plasmids described in the previous paragraph were combined and annealed for 16 h at 37 °C. The formation of the figure-eight structure was first analyzed by fractionation on agarose gel following restriction digestion with *Bgl*II. Fractionation of the annealing products on agarose gel revealed two bands of relatively equal amounts of DNA. The lower one migrated at the same distance of the untreated single plasmid, whereas the second band migrated at a higher molecular weight. This could be attributed to the figure-eight structure

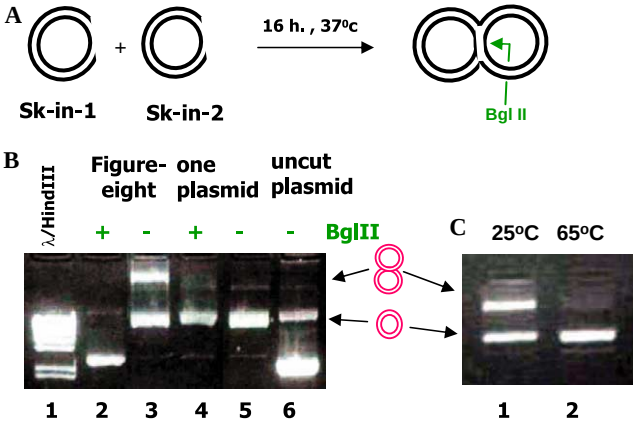

Fig. 3. Formation of the figure-eight structure. (A) Schematic representation of the annealing step. (B) Analysis of the figure-eight structure. When digested with *Bgl*II, the figure-eight structure was disrupted to produce the linear plasmids (lane 2). Analyzing the hybridization product revealed a significant amount of the figure-eight structure (lane 3). Incubating only one plasmid did not result in cleavage by *Bgl*II (lanes 4 and 5). When the plasmid was digested with *Bgl*II before the release of the single-stranded fragment, a linearized plasmid was produced (lane 6). (C) The figure-eight structure was disrupted by heating to 65 °C. A schematic representation of the linearized plasmids, a single plasmid, and the figure-eight structure is presented between (B) and (C).

(Fig. 3B, lane 3, and Fig. 3C, lane 1). Following digestion with *Bgl*II, the two bands were converted into a single one migrating at a size of 3 kb, resembling a linearized single plasmid (Fig. 3B, lane 2). This result indicated that the *Bgl*II site became double-stranded and available for digestion by the restriction enzyme as a result of the figure-eight structure formation. In addition, the result that both the single and figure-eight bands were converted into a linearized plasmid suggested that an equilibrium relationship existed between the two forms. Under this equilibrium, when the figure-eight structure is broken by digestion with *Bgl*II, more of the single plasmid population is converted to the figure-eight form. As a control, a single plasmid population Sk-in-1 that cannot produce the figure-eight structure was incubated with the enzyme and found not to be cleaved by *Bgl*II (Fig. 3B, lanes 4 and 5). In addition, heating the figure-eight solution to 65 °C for 10 min, followed by rapid cooling on ice, resulted in the dissociation of the two plasmids, as expected, due to the dissociation of the annealed 18-base segments (Fig. 3C). Taken together, this analysis demonstrated that the figure-eight structure was formed at a relatively high yield.

Construction of figure-eight structure using bacteriophage M13 single-stranded DNA

A second approach for the construction of the figure-eight structure using single-stranded DNA was also developed. For this, we made use of the bacteriophage M13-derived vectors M13mp18 and M13mp19 [12,13], which may be obtained in two forms. The first form is a double-stranded circular molecule of 7250 nt maintained in the bacteria cells as a plasmid. Therefore, it is available for genetic engineering manipulations, including adding/deleting DNA fragments by making use of the restriction enzyme sites. The second form is a single-stranded DNA obtained when the bacteria produces the bacteriophage form. The M13 vectors Mp18 and Mp19 are identical in the nucleotide sequences except in the multicloning site, which is composed of 48 nt and is complementary between the two vectors [13]. First, the two forms of bacteriophage DNA, single-stranded and double-stranded DNA, were produced and analyzed. Bacteriophage particles were collected from the growth medium for the production of single-stranded DNA, whereas the double-stranded DNA was purified from the bacteria cells. The quality of single- and double-stranded DNA preparations was analyzed on agarose gels following digestion with either mung bean exonuclease, which digested only single-stranded DNA, or

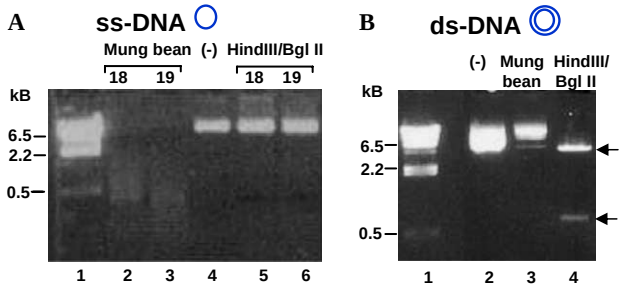

Fig. 4. Analysis of the single-stranded M13 molecules. The single-stranded (A) or double-stranded (B) forms of M13 were digested with mung bean nuclease (digesting exclusively single-stranded DNA) (lanes 2 and 3 in (A) and lane 3 in (B)) or with restriction enzymes *Hin*dIII and *Bgl*II (digesting only double-stranded DNA) (lanes 5 and 6 in (A) and lane 4 in (B)). The products were resolved on agarose gel alongside λ/*Hin*dIII size markers (lane 1 in (A) and (B)). (-), indicates untreated DNA.

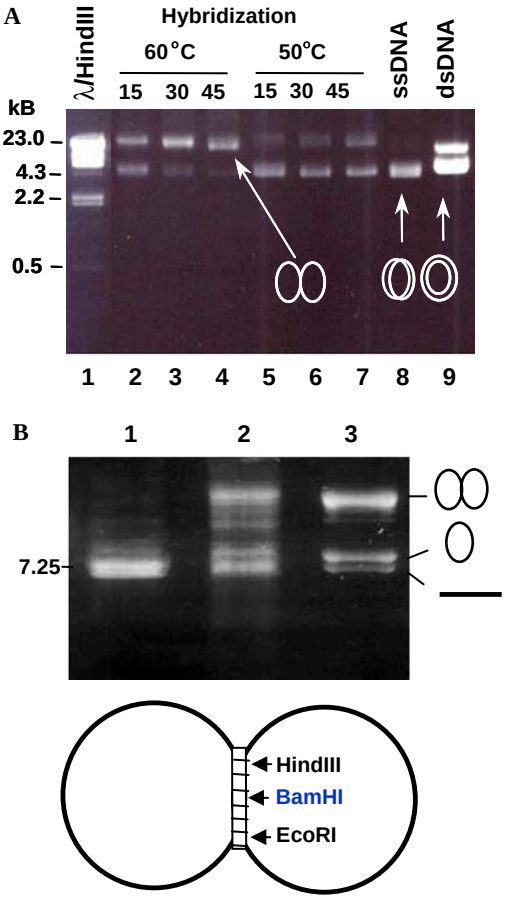

Fig. 5. Formation of figure-eight structure by annealing two circular M13 DNA molecules. (A) A single-stranded circular DNA molecule was produced using the system of M13mp18 and M13mp19. The two vectors are identical except for a sequence of 48 bases that is complementary. The two molecules were mixed and allowed to hybridize at 60 or 50 °C for the times indicated in the figure. Analysis was done by fractionation on 0.8% agarose gel and ethidium bromide staining. In the lane marked dsDNA, a supercoiled double-stranded form of this vector was loaded onto the gel. (B) Analysis of the figure-eight structure of M13 by restriction endonucleases. The figure-eight structure of M13 was digested with *Hin*dIII and *Eco*RI (lane 1) or *Bam*HI (lane 2) or was uncut (lane 3). Whereas *Hin*dIII/*Eco*RI disrupted the figure-eight structure, indicating that double-stranded DNA was indeed formed at the location of sites for these enzymes, digestion with *Bam*HI did not disrupt the figure-eight structure.

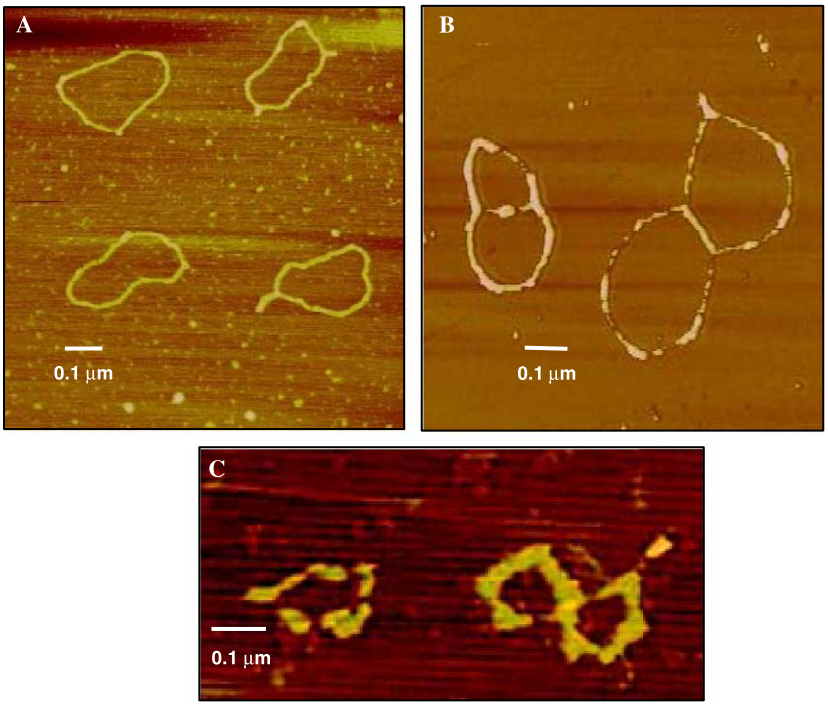

Fig. 6. Visualizing the figure-eight structure using AFM. Samples of the double-stranded plasmids were prepared by cleaving mica with adhesive tape and depositing 0.2 ml of DNA solution, followed by 4 min of rinsing with H_2O and drying with a stream of argon. Samples of the single-stranded M13 DNA were prepared as described in Materials and methods. Samples were imaged with AFM in the tapping mode in air using RTESP (silicon) cantilevers. Fields of less than 10 μm were scanned at 1 Hz per scan line. Typical AFM images of the single (A) and figure-eight (B) structures of the Sk plasmids and those of the single-stranded M13 DNA (C) are presented. In (C), the structure on the left is probably a single M13 molecule, whereas that on the right is a figure-eight structure composed of two M13 molecules. The bar indicates a scale of 0.1 μm.

restriction enzymes *Hin*dIII and *Bgl*II, which cut only the double-stranded DNA. Fig. 4A shows that the single-stranded DNA was indeed sensitive to the mung bean nuclease treatment (lanes 2 and 3) but was completely resistant to the restriction enzymes (lanes 5 and 6). Fig. 4B shows the same assays performed on the double-stranded DNA form of M13mp18. Here, the DNA was resistant to mung bean ribonuclease (lane 3) but was digested completely by the restriction enzymes (lane 4). This analysis demonstrated conclusively the intactness and purity of the single- and double-stranded M13 DNA preparations.

The two single-stranded DNA molecules were allowed to hybridize at 50 and 60 °C. As can be seen in Fig. 5A, following analysis on agarose gel, there appeared a slower migrating band whose amount was increased during the incubation time and reached approximately 80 to 90% following 45 min at 60 °C (Fig. 5A). This band probably indicates the figure-eight structure formed by the hybridization of the M13mp18 and M13mp19 single-stranded DNA molecules. To verify that this is actually the case, the molecules were analyzed by digestion with restriction enzymes. Because these enzymes cut only when double-stranded DNA is present, successive cleavage implies the formation of this form. When the DNA was cleaved with *Hin*dIII and *Eco*RI, only one band corresponding to linear molecules of approximately 7 kb was observed (Fig. 5B, lane 1). Because the restriction sites of these enzymes are located near the border of the 48 nt region forming the double-stranded DNA, this result demonstrated that the hybridization indeed occurred, creating the double-stranded DNA, and that both enzymes cut the DNA, resulting in two single-stranded DNA molecules that could not be hybridized to each other anymore. However, cleavage with *Bam*HI, whose site is located in the middle of the hybridization sequence, did not result in separation of the two circular molecules (Fig. 5B, lane 2). The simplest explanation for this result is that although it was cleaved by *Bam*HI, the remaining double-stranded DNA sequences on each side of the cleavage (~24 nt) kept the two circular molecules together. Taken together, the results of this experiment show that a figure-eight DNA structure composed of two circular single-stranded DNA molecules that hybridized together was established. Therefore, we next aimed at visualizing the figure-eight structure using atomic force microscopy (AFM).

Visualizing the figure-eight structure using AFM

The single plasmids and the figure-eight constructs were spread onto freshly cleaved mica and visualized using AFM. The single plasmid could be observed

clearly as a circular structure of approximately 0.16 μm diameter and 1 μm circumference, as is expected of a 3000 bp circular DNA plasmid (Fig. 6A). When the figure-eight structure construct was analyzed, many figure-eight structures could be observed (Fig. 6B). The connection site of the two plasmids is approximately 0.1 μm long, which is significantly longer than the length calculated for 18 nt of the single-stranded region of plasmids (0.006 μm). Because we observed this phenomenon in most of the figure-eight structures that were analyzed, it is possible that the attachment region of the plasmids extends further than the single-stranded region. However, another possibility is that the extended connection length was created during the preparation of the sample for AFM analysis.

When the figure-eight structure of the single-stranded DNA constructs was analyzed by the AFM technique, we observed many "connected circles" of DNA (similar to the plasmids described above). Fig. 6C presents an example of such a structure (right side) as compared with a single circled structure (left side). However, these circles were much shorter than the expected length of a DNA molecule of approximately 7000 bases and probably reflected the complication and formation of secondary structures often observed for single-stranded DNA [14,15]. Nevertheless, the formation of a figure-eight structure could be detected at significant portions of the circled structures (Fig. 6C).

Together, the gel and AFM analysis demonstrated the successful formation of the figure-eight structure harboring the two attached double- or single-stranded plasmids.

Conclusions

Molecular self-assembly offers a means of spontaneously forming complex and well-defined structures from simple components. This study has demonstrated that long single- or double-stranded circular DNA molecules can produce figure-eight structures. Manipulation of the basic circular DNA structures could produce a family of scaffold variants whose functions could vary, for example, by the addition of other materials, such as electronic devices, at uniquely defined locations. DNA nanostructures like the one reported here can, therefore, be regarded as devices that use molecular recognition to produce high-order, including three-dimensional, structures [16–18]. Demonstrating the formation of the figure-eight DNA structure is a step toward making more sophisticated devices for molecular electronic and nanotechnology applications.

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