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Long-Range Assembly of DNA into Nanofibers and Highly Ordered Networks Using a Block Copolymer Approach

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Abstract: A simple method to introduce the long-range order achieved by block copolymers into DNA structures is described. This results in the hierarchical assembly of short DNA strands into a new one-dimensional material, with high aspect ratio and the ability to further align into highly ordered surfaces over tens of micrometers. Fibers derived from biological materials have a wide range of potential applications, such as scaffolds for nanowires and one-dimensional (1D) materials, templates for tissue growth, and ligand display tools for multivalent biological interactions. Fibers derived from short DNA strands are an attractive class of materials, as they combine long-range 1D ordering with the programmability of DNA, and its ability to undergo structure switching with specifically added DNA strands. Here, we present the first examples of long fibers self-assembled from short (10−20 base-pairs), blunt-ended DNA strands. This was accomplished by covalently attaching a dendritic oligoethylene glycol (OEG) unit to a DNA strand to form a dendritic DNA molecule (D-DNA). Hybridization of this unit with complementary DNA creates a block copolymer/double-stranded DNA architecture, which readily undergoes self-assembly into long fibers upon the addition of a selective solvent. These fibers can further align into parallel rows, to yield highly ordered micrometer-sized surfaces. We demonstrate that a DNA nanotechnology motif, a three-helix DNA bundle, can also be readily induced to form long fibers upon incorporation of D-DNA. Thus, this provides a straightforward method to introduce hierarchical long-range ordering into DNA motifs, simply through hybridization with short D-DNA strands.

Introduction

DNA has recently emerged as a highly effective template for the programmable assembly of nanostructures.[1−4] With desirable properties such as high base-pairing fidelity, designable sequence, ease of synthesis, high aspect ratio, and a rich toolbox for functionalization, DNA is a unique nanoconstruction tool.[1−4] DNA can position materials with precision into periodic or aperiodic structures, using intermolecular interactions that can be addressed even after the assembly takes place.[1−4] However, a significant challenge is to create DNA nanostructures with long-range order, as sequence engineering significantly increases in complexity, and errors arise in the assembly when the structure increases in size.[5]

An extensively used method to create nanostructures with long-range order involves the self-assembly of amphiphilic block copolymers, through microphase separation of dissimilar blocks. Demixing is energetically favorable, while macroscopic separation is hindered by the covalent bond between the different blocks.[6−8] Microphase separation leads to a number of predictable morphologies that display very long-range ordering. Applications of block copolymer systems range from drug delivery to nanoelectronics.[9] However, conventional block copolymer assembly does not present the opportunity to achieve programmability at the molecular level.

A particularly attractive goal would be to combine the two assembly methods, DNA and block copolymer architecture, to form DNA structures that would have molecular programmability while achieving long-range order. Herein, we report the synthesis and self-assembly behavior of a dendritic DNA (D-DNA) molecule composed of an oligoethylene glycol (OEG) dendron covalently attached to a single strand of DNA (Figure 1). Upon hybridization of complementary D-DNA strands, a D-DNA triblock copolymer is constructed (Figure 2). Addition of a selective solvent, such as acetonitrile, leads to the self-assembly of these double-stranded D-DNA building blocks into long fibers. Even with short DNA strands (10 or 20 base-pairs), these one-dimensional structures extend over many micrometers and can further align in parallel rows, into highly periodic surfaces over tens of micrometers in size. Single strands of D-DNA can even mediate the self-assembly of unmodified complementary DNA, as well as other DNA nanotechnology motifs into fibers with long-range order and high aspect ratio. Thus, this introduces a highly simplified method to achieve long-

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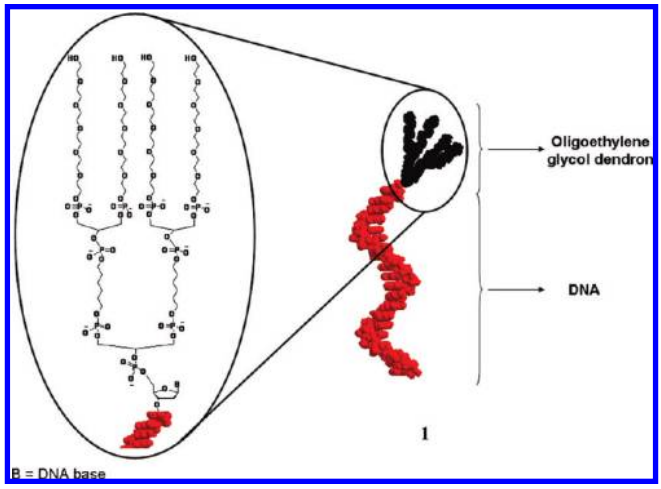

Figure 1. D-DNA **1** chemical structure.

range order into DNA structures, merely by hybridization with short D-DNA strands.

Long-range 1D-ordering with biological structures has been an area of intense research. Some peptides and proteins such as collagen[10] and amyloid[11,12] have a natural tendency to organize into fibers with high aspect ratio and hierarchical ordering. Amphiphilic peptides have been developed to assemble into long fibers with a number of biological and materials applications.[13,14] In addition, very long DNA strands (tens of thousands of base-pairs) can be induced to form fibers using a variety of methods.[15−17] However, to our knowledge, long-range ordering of simple, short DNA strands without the use of sticky-end cohesion has not been previously reported. DNA fibers would have a number of applications, from gene delivery, to programmable scaffolds for tissue engineering, to templates for nanowires and dynamic, addressable 1D nanostructures.

Results and Discussion

Synthesis and Characterization of Dendritic DNA (D-DNA). The synthesis of D-DNA building blocks was performed using a divergent approach.[18] A DNA strand containing 20 bases was grown on a DNA synthesizer, followed by stepwise addition of branching phosphoramidites and a hexaethylene glycol phosphoramidite derivative (referred to as OEG in this Article), to make the final D-DNA structure (Figure 1).[36] A maximum of four OEG chains could be efficiently attached to short (10−30 bases) DNA strands. The yield of the four-armed dendron was further optimized upon addition of a spacer molecule containing six carbons between branching units to yield D-DNA **1** (Figure 1). All of the D-DNA strands synthesized were isolated and purified by gel electrophoresis and size exclusion chromatography. The D-DNA products were then characterized by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry[19] and gel electrophoresis under denaturing conditions.[36]

Next, D-DNA strands were hybridized with complementary unmodified DNA strands or with complementary D-DNA strands in 10 mM sodium phosphate buffer (pH 7.3). The hybridization of D-DNA duplexes containing one or two four-arm dendrons per duplex was quantitative as ascertained by gel electrophoresis.[36] The D-DNA duplex was further characterized by circular dichroism (CD) spectroscopy and thermal denaturation experiments. The CD spectrum of the D-DNA duplex has dichroic peaks similar to those of an unmodified DNA duplex, indicating that D-DNA duplex maintains a B-DNA form.[20,36] Thermal denaturation experiments show that the D-DNA duplex has a sigmoidal curve shape similar to that of an unmodified DNA duplex, showing that the cooperativity between complementary strands is maintained, and a melting temperature of only 3 °C below that of the unmodified DNA duplex indicates that the dendron does not significantly affect duplex stability and binding cooperativity.[36]

Self-Assembly of D-DNA Duplexes in Organic Solvent. DNA has been conjugated to a long polypropylene oxide (PPO) chain to form block copolymers that self-assemble in aqueous solution into spherical micelles, with a PPO core and DNA strands on their exterior.[21] The D-DNA structures generated here contain a small, dendritic, and monodisperse OEG moiety attached to a DNA strand.[22] Upon addition of an organic solvent to the aqueous solution, microphase separation is expected to create reverse micellar morphologies containing surface exposed OEG dendrons, as these are expected to be more soluble in the organic medium than the DNA strands.

The assembly of D-DNA was studied by tapping mode atomic force microscopy (AFM). The samples were prepared by hybridizing 0.05 nmol of complementary D-DNA strands in 5 μL of 10 mM sodium phosphate buffer (pH 7.3) to form D-DNA **2** duplex, followed by dropwise addition of 15 μL of anhydrous acetonitrile. This solution was drop-cast on a freshly cleaved mica surface and analyzed by AFM within 24 h. At acetonitrile concentrations above 50%, well-defined fibers were observed, with the most abundant fibers forming at 75% acetonitrile/buffer solution.[36] As shown in Figure 2a, these fibers extend over many micrometers in length and account for almost all morphologies observed. AFM height measurements indicate that the fibers are somewhat embedded in the substrate, with an individual fiber average height of 1.86 ± 0.46 nm (Table 1). As well, fiber bundles with average heights ranging from 2 to 8 nm were observed.[36] It is of note that this organization of short D-DNA strands into fibers occurs in sodium phosphate buffer/acetonitrile solutions, with no divalent or trivalent cations added.[37]

CD experiments of DNA and D-DNA duplexes indicate that the double-stranded character is maintained at 75% acetonitrile solvent composition.[36] Control experiments showed that double-stranded DNA that was not modified with OEG dendrons did not produce fibers from acetonitrile/aqueous buffer solution.

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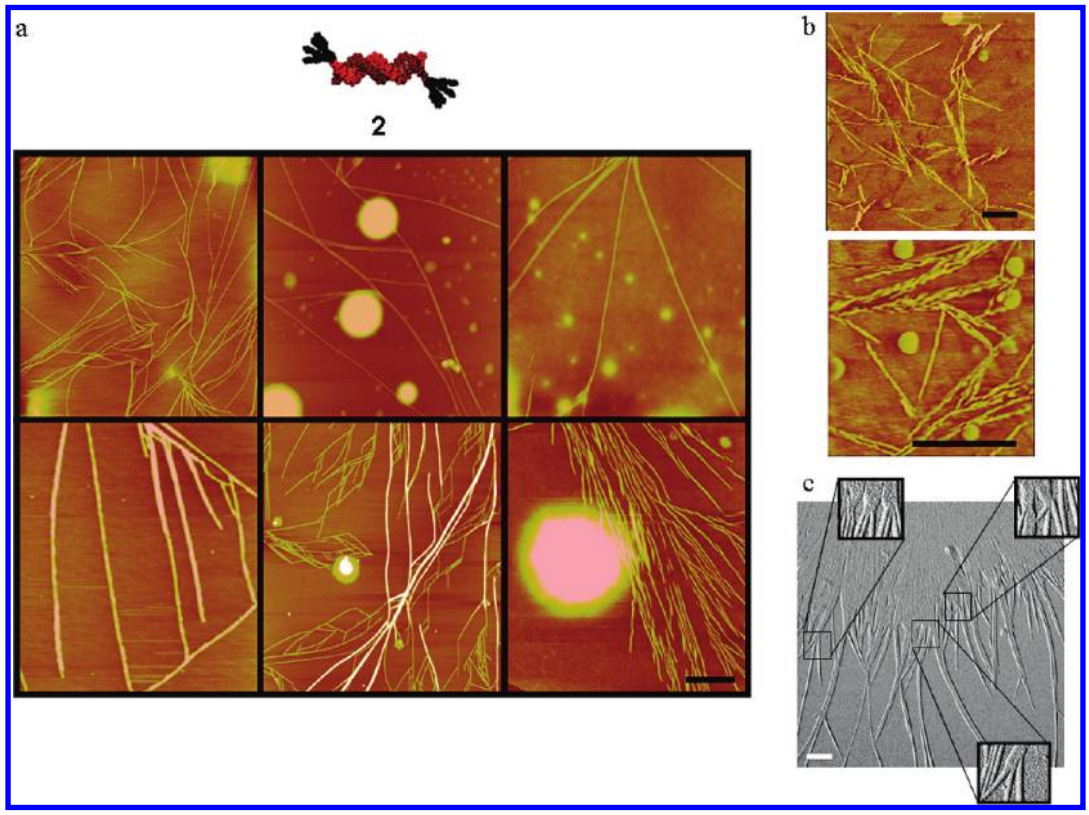

Figure 2. Self-assembly of D-DNA **2** prepared from 75% acetonitrile/buffer solution (sequence: (5′-(OEG)-TGCATAGCAATGCCTCGAAG-3′, and 5′-(OEG)-CTTCGAGGCATTGCTATGCA-3′). (a) Tapping mode AFM images on mica (bar is 1 μm). (b) Tapping mode AFM images on HOPG (phase image, bar is 500 nm). (c) Platinum shadowing TEM image. Insets show braiding of the fibers (bar is 0.2 μm).

Table 1. Average Individual D-DNA Fiber Heights

D-DNA no.	average height (nm)
2	1.86 ± 0.46
3	1.78 ± 0.35
4	1.83 ± 0.33
5	1.90 ± 0.33
6	2.16 ± 0.33
7	1.83 ± 0.29
8	2.18 ± 0.60

Interestingly, the single-stranded form of D-DNA does not show such self-assembly into fibers, pointing to the role of duplex rigidity and/or π-stacking in this assembly.[36]

Structural Variation. A number of modifications to the D-DNA building block also resulted in fiber formation. Decreasing the length of D-DNA duplex strands from 20 (D-DNA **2**, Figure 3a) to 10 (D-DNA **3**, Figure 3b) base-pairs also resulted in fiber formation from acetonitrile/buffer; however, increasing the DNA length to 30 base-pairs per dendron gave ill-defined morphologies,[36] consistent with the requirement of an optimal DNA:OEG ratio for fiber formation. In addition, decreasing the number of OEG arms from four to two arms per DNA strand also resulted in long fibers by AFM (D-DNA **4**, Figure 3c); however, further decrease in the number of arms from two to one arm per DNA strand suppressed fiber formation,[36] again consistent with microphase separation no longer being energetically favorable at this DNA:OEG ratio. Hybridization of D-DNA with an unmodified complementary DNA strand produced an asymmetric duplex with one dendron on only one of its extremities (D-DNA **5**, **6**, and **7**, Figure 3d−f). Interestingly, this architecture also gave well-defined fibers (Figure 3d−f). These results suggest the usefulness of D-DNA single strands as tools to mediate the self-assembly of unmodified DNA motifs into structures of high aspect ratio. Average AFM heights for individual D-DNA fibers (structures **2**−**7**) were around 2 nm; these measurements are summarized in Table 1.

Assembly of D-DNA into Fibers on Other Substrates and in Solution. It is of note that the absence of divalent or trivalent cations in the buffer medium is expected to prevent strong interactions of the DNA duplexes with the negatively charged mica surface; thus the observed fiber structures are not likely to be merely a result of mica-directed ordering effects.[37] Nevertheless, to ascertain that the assembly is not a surface-directed phenomenon, we investigated fiber formation at 75% acetonitrile concentration by AFM on highly ordered pyrolytic graphite (HOPG), by TEM on carbon-coated grids with a negative stain, and in solution by dynamic light scattering (DLS). The D-DNA duplex also formed well-defined fibers on the hydrophobic HOPG surface (Figure 2b). Moreover, these fibers appear to be helical, unlike the fibers observed on the mica surface. Fibers were also observed by TEM on carbon-coated copper grids that were negatively stained with methylamine vanadate ("Nanovan"). These fibers appeared to be more bundled on this hydrophobic surface than on the mica surface in the AFM experiment. The average width of these fibers was 16.9 ± 3.2 nm.[36] DLS experiments indicated that D-DNA fibers not only assemble on surfaces, but are also present in solution. The measured average hydrodynamic diameter (D_H) at 90° was 1.7 μm, and it varied at different angles, as is expected for nonspherical morphologies in solution.[36]

Light scattering experiments were also carried out in an effort to assess the kinetics of D-DNA assembly in 75% acetonitrile solution.[36] The scattering intensity for D-DNA solution dramatically increased immediately upon acetonitrile addition, and then

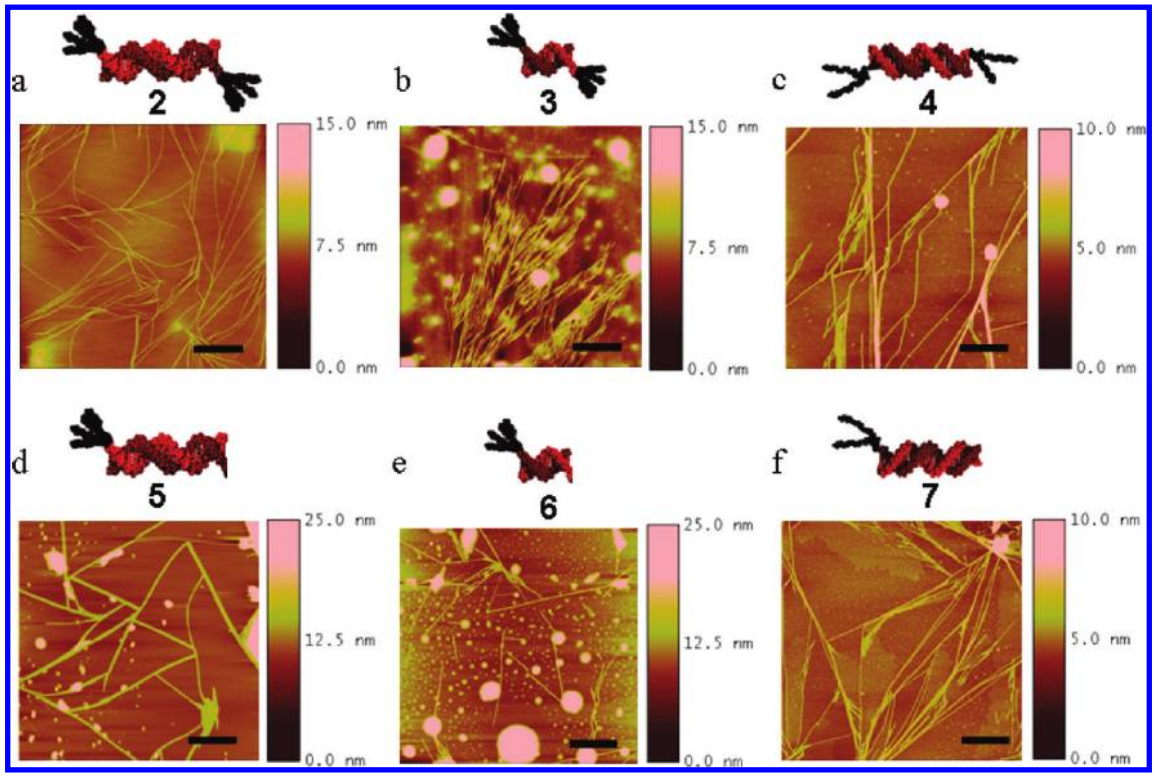

Figure 3. Self-assembly of modified D-DNA duplex prepared from 75% acetonitrile/buffer solution. AFM of D-DNA duplex containing (a) 20 base-pairs (**2**) and (b) 10 base-pairs (**3**) at 75% acetonitrile concentration and (c) AFM of D-DNA duplex containing 20 base-pairs and two OEG arms (**4**). AFM of asymmetric D-DNA duplex containing only one OEG dendron at one of its extremities, with (d) 20 base-pairs (**5**) and (e) 10 base-pairs (**6**) at 75% acetonitrile concentration and (f) AFM of D-DNA duplex containing a dendron with only two OEG arms (**7**) (bar is 1 μm).

slowly decreased over a period of a few hours.[36] This result is consistent with rapid association of D-DNA into higher-order structures, followed by precipitation of these assemblies over time.

There are a number of possible mechanisms that can be invoked for individual D-DNA fiber growth (Figure 4). In all cases, assembled structures are expected to contain the DNA block in the core of the fiber, while the OEG dendrons, which are more soluble in acetonitrile, would reside at the exterior. Figure 4a shows one likely mechanism, in which the D-DNA building blocks are assembled in a parallel fashion to the growing direction of the fiber, in an end-to-end stacking arrangement. Short, unmodified DNA duplexes have been shown to similarly stack in an end-to-end fashion to form soft liquid crystalline phases.[23] In comparison, the D-DNA duplexes here are surrounded by OEG dendrons, which can possibly sterically prevent them from forming large liquid crystalline domains, thus confining them to 1D-fiber morphologies. Once a single fiber is formed, braiding or bundling of two or more fibers may occur to further protect the DNA core from the organic solvent (Figure 4a).[24,25] Alternatively, fiber formation may occur with the D-DNA strands oriented in a perpendicular fashion to the fiber direction, with a parallel, side-by-side arrangement (Figure 4b). Considering that the buffer used only contains monovalent ions, this parallel arrangement may be hindered by electrostatic repulsion between the closely packed DNA duplexes. A third assembly possibility is the formation of nanoribbons (Figure 4c); however, ribbon assembly would expose the DNA core to the acetonitrile solvent to a greater extent than the two previous mechanisms.

The measured heights for individual D-DNA (**2** and **3**) fibers are around 2 nm (Table 1), while bundles of fibers range from 2 to 8 nm in height.[36] Because the sample is somewhat embedded, and due to sample compression by the AFM tip, all recorded heights are most likely smaller than their actual values. Nevertheless, these individual fiber heights are close to the width of a DNA duplex (~2 nm), and, as such, they are more consistent with the end-stacking mechanism of Figure 4a. If the mechanism in Figure 4b were at play, a single fiber of D-DNA **2** (20 base-pairs) would be expected to show a height of 11 nm (AMBER force field molecular modeling, Hyperchem), and bundles would be even higher. Instead, single fibers of D-DNA **2** show average heights of ~1.86 nm, and fiber bundles are no higher than 8 nm. In addition, there is no observable height doubling upon going from D-DNA **3** (with 10 base-pairs in the duplex, height 1.78 ± 0.35 nm) to D-DNA **2** (20 base-pairs, height 1.86 ± 0.46 nm), which would be expected for the mechanism of Figure 4b. Thus, the AFM height analysis of these fibers is more consistent with the end-to-end stacking mechanism of Figure 4a.[26]

In an effort to differentiate between the different mechanisms of individual fiber formation, we designed a D-DNA building block containing a two-base sticky-end overhang at each side of the duplex (Figure 4e (i)).[36] The rationale behind this design is that a two-base sticky-end would stabilize fiber formation in an end-to-end D-DNA stacking (Figure 4e (ii)). On the other

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(26) The fibers are somewhat embedded and the tip is compressing the sample, and thus the measurements are approximate.

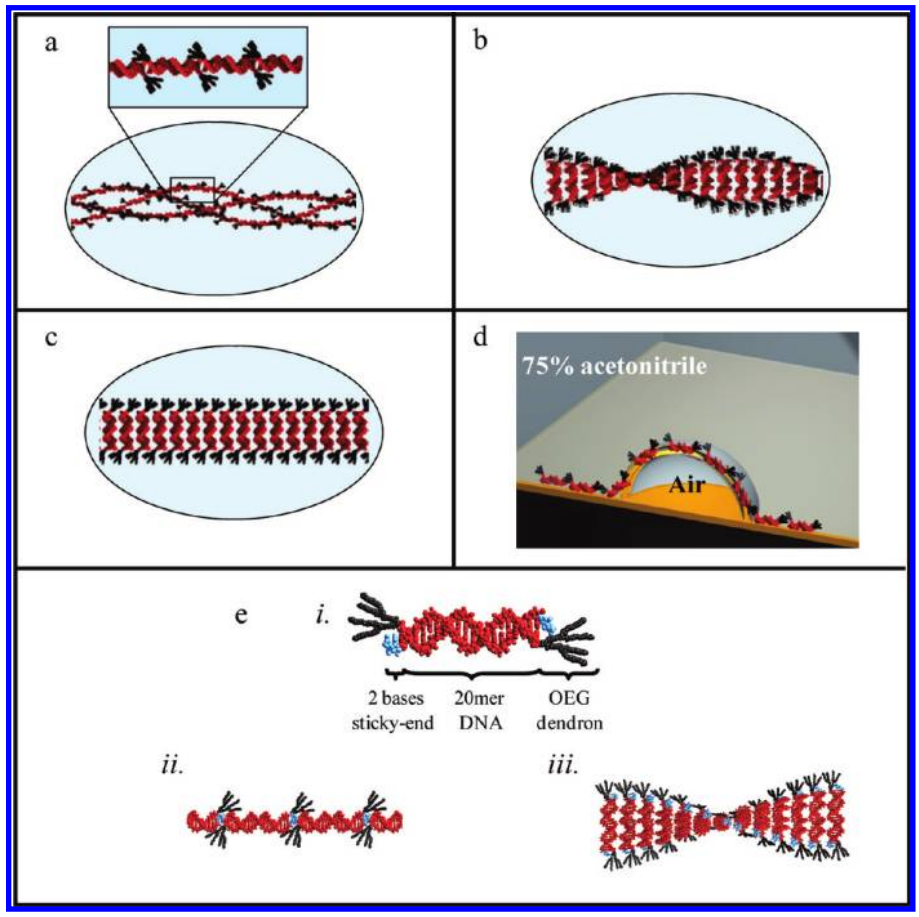

Figure 4. Possible D-DNA assembly mechanisms. (a) D-DNA duplexes stacked in an end-to-end fashion forming fibers that eventually braid onto one another. (b) D-DNA duplexes stacked in a side-by-side arrangement perpendicular to the fiber direction. (c) D-DNA stacked side-to-side forming nanoribbons. (d) D-DNA arranging in a "Janus"-type morphology at the interface of air bubbles. (e) D-DNA 20 base-pair duplex with 2 base sticky-ends (blue color) at each extremity (sequence: 5′-(OEG)-TGCATAGCAATGCCTCGAAGTG-3′, and 5′-(OEG)-CTTCGAGGCATTGCTATGCACA-3′). (i) AMBER force-field model (Hyperchem), (ii) fiber assembly end-stacking mechanism with sticky-end D-DNA, and (iii) fiber assembly mechanism with DNA strands perpendicular to the fiber direction, with sticky-end D-DNA.

hand, if the DNA strands are perpendicular to the fiber direction (Figure 4e (iii)), then the sticky-ends would be too short and therefore sterically inaccessible to hybridize across two fibers, and the hybridization of two bases would provide insufficient enthalpic gain to connect the fibers together. Thus, increased fiber formation should be suggestive of mechanism of Figure 4a. Both AFM and light scattering experiments showed increased fiber formation for the sticky-ended D-DNA.[36] The light scattering intensity and the hydrodynamic diameters were greater for the sticky-end DNA assembly, and the light scattering intensity decreased at a much faster rate than for blunt-ended DNA.[36] This is consistent with increased aggregation and possible fiber stabilization with the aid of sticky-end cohesion, likely through the end-to-end stacking mechanism of Figure 4a.

Effect of Gas Bubbles on Assembly of D-DNA and Formation of Ordered Networks. It has been previously reported that aqueous binary mixtures contain stable air bubbles[27,28] and/or microdomains[29−31] of solvent in solution. The mechanism of fiber formation from D-DNA may be influenced by the interfaces of these heterogeneous domains. In the presence of an interface, one can imagine D-DNA building blocks assuming a "Janus"-type morphology during fiber formation, with the DNA facing the more hydrophilic side of the interface and the OEG dendrons facing the more organic medium (Figure 4d).

We thus investigated how the presence of gas bubbles in 75% acetonitrile aqueous solution affected fiber formation. Nitrogen gas was bubbled into 75% acetonitrile aqueous solutions of D-DNA duplexes (both 10 and 20 base-pairs long). For both 10 and 20 base-pairs D-DNA (**3** and **2**), a larger amount of fibers was observed by AFM upon addition of nitrogen gas, with most fibers residing at the gas−solvent interface. This either indicates that the fibers adsorb efficiently on these bubbles after their formation or that the presence of these interfaces may play a role in nucleating fiber formation (Figure 5a and b).

Surprisingly, removal of gas bubbles from the D-DNA solution did not suppress fiber formation. Freeze−pump−thaw cycles were performed until all visible bubbles were removed, and this solution was quickly drop-cast on a mica surface. This now resulted in highly aligned and uniform fibers on the mica surface, forming extended surfaces of greater than 10 μm in size. Figure 5c is an AFM micrograph obtained from D-DNA **2** duplex from a degassed 75% acetonitrile solution. The fibers obtained seemed to be braided, with an average height of 3.82 ± 0.81 nm, and with a crest distance of 25 nm. The width

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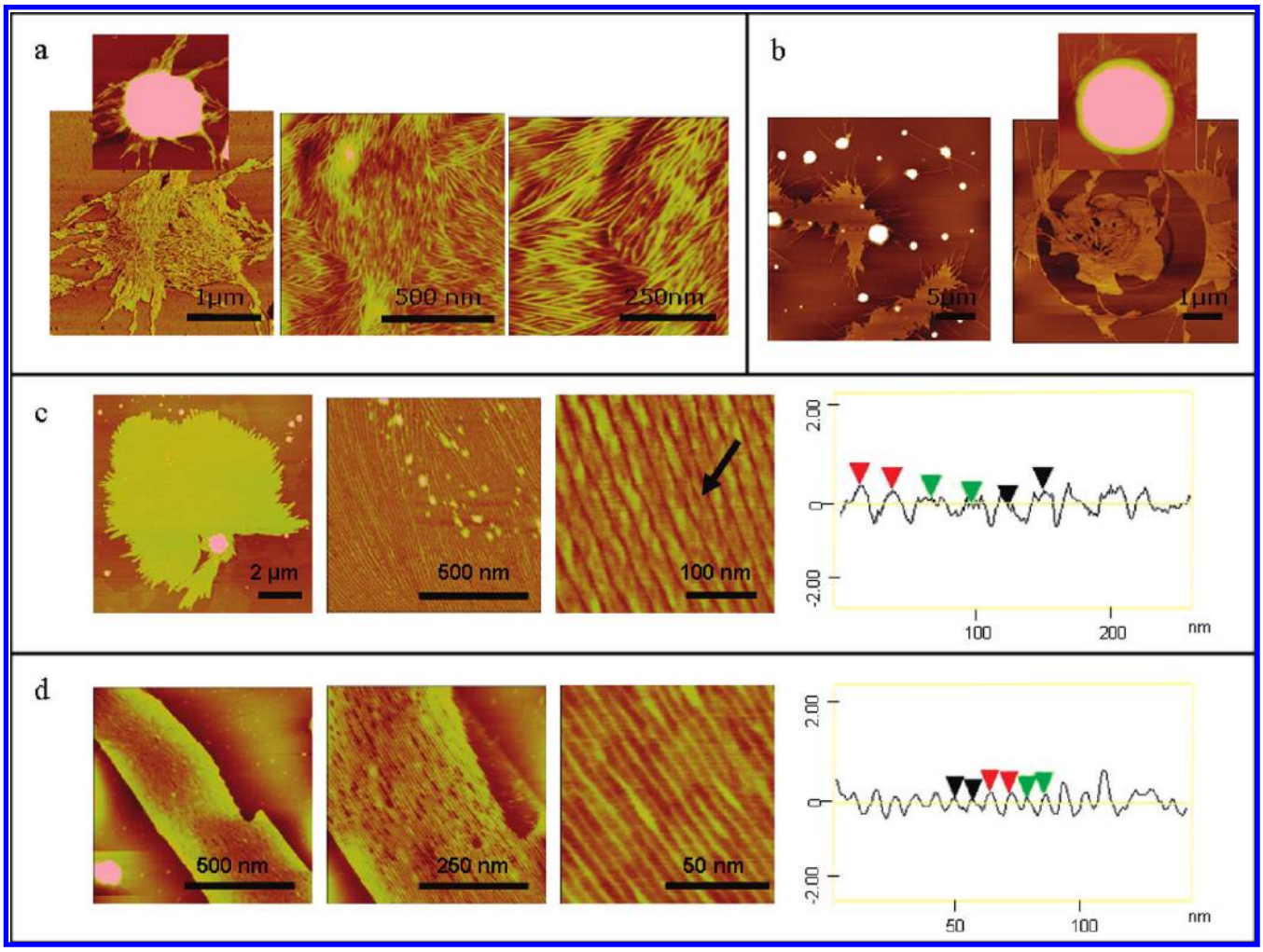

Figure 5. Role of gas bubbles in D-DNA assembly. AFM images of 75% acetonitrile solution containing D-DNA duplexes with (a) 20 and (b) 10 base-pairs upon addition of nitrogen to solution. AFM images of 75% acetonitrile solution containing D-DNA duplexes with (c) 20 and (d) 10 base-pairs upon degassing and removal of bubbles from solution.

measurement for D-DNA **2** fibers may correlate with the width of three or more fibers braiding onto one another, assuming the end-to-end morphology of Figure 4a. The arrow in Figure 5c points to a branch in which one of the fibers splits into two, and these branches in fiber direction appear to be a dominant feature in the sheets from D-DNA **2**. Thus, each aligned fiber is likely formed by individual strands braiding with one another. Similar results were obtained for fibers from D-DNA **3**, but braiding was not evident using AFM. These aligned fibers had a width of 8 nm, which may correlate with the width of a single fiber (Figure 5d).[32] We hypothesize that once gas bubbles with curved surfaces were removed, the fibers are able to align well on the atomically flat mica surface, further reducing the exposure of the DNA strands to the organic medium. This hierarchical assembly has been previously reported with amyloid fibrils, as well as cylindrical micelles from synthetic triblock copolymers, which associate into higher-order structures by braiding with one another.[23,24]

The braiding of the fibers formed from D-DNA building blocks was also observed by transmission electron microscopy (TEM) platinum shadowing experiments. Most of the fibers in the D-DNA **2** duplex split into three or more individual fibers, supporting the braiding mechanism of fiber formation (Figure 1c). Some braiding was also observed in the D-DNA **3** building block, but not to the same extent as D-DNA duplex with 20 base-pairs.[36]

Self-Assembly of Other DNA Motifs into Nanofibers Using D-DNA. DNA has been used to form a large number of highly organized nanostructures.[1−5] We were interested in investigating whether DNA nanotechnology motifs could undergo hierarchical assembly when attached to the OEG dendrons. We assembled a dendritic three-helix DNA bundle **8** containing three D-DNA single strands hybridized to one another by three linking strands.[33] Structure **8** was formed in near quantitative yield, as assessed by gel electrophoresis.[36] In this case as well, **8** assembled into fibers in 75% acetonitrile solutions (Figure 6), with greater average heights (2.18 ± 0.60 nm) than those of the simple D-DNA assemblies (**2** and **3**). Thus, incorporation of D-DNA strands into DNA nanotechnology motifs is a simple

(32) Assuming end-to-end stacking (Figure 4a) and taking into consideration the width of DNA duplex and OEG dendron.

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(35) The fibers are formed from acetonitrile/water solutions. For biological applications in aqueous media, they could be covalently joined using standard reactions (e.g., disulfide links, "Click" reactions, or phosphodiester ligations), or they could be used as preassembled surfaces.

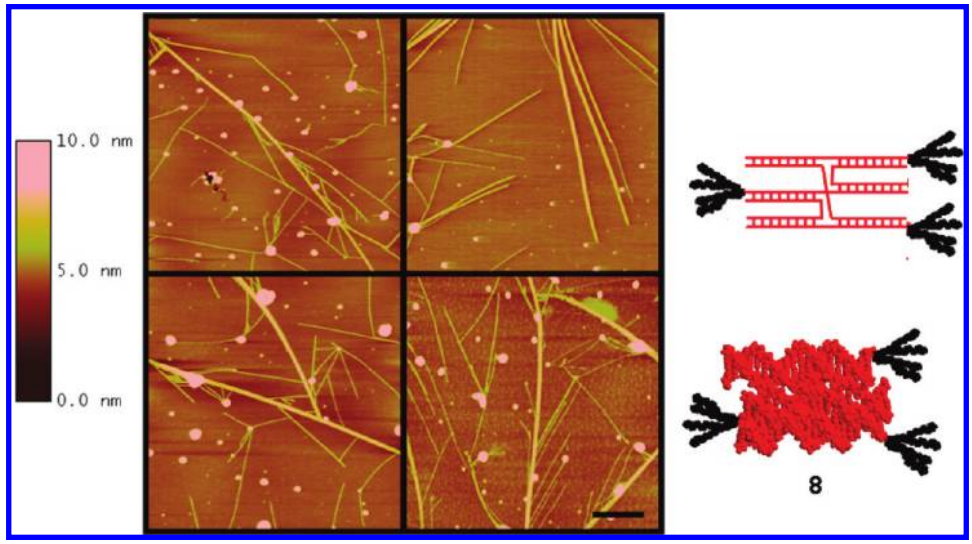

Figure 6. Three-helix bundle assembly. AFM of **8** on mica assembled from a 75% acetonitrile solution (bar is 1 μm).

method to endow these structures with the ability to undergo self-assembly into long-range ordered structures.

Conclusions

The results herein reported describe a highly simplified method to introduce the long-range order achieved by block copolymers into DNA structures. This results in long fibers that retain the double helical character of DNA and can thus utilize its ability to programmably position materials, as well as its addressable, dynamic, and molecule-responsive character. D-DNA molecules containing a short DNA strand (10−20 bases) can be readily generated by automated solid-phase methods. Hybridization of a DNA strand (even unmodified) with these D-DNA molecules induces its assembly into long fibers, which further align into highly ordered micrometer-sized surfaces. We also demonstrate that D-DNA can induce a DNA three-helix bundle to undergo assembly into fibers, thus extending the assembly potential of DNA nanotechnology into long-range structures.

On a fundamental level, short DNA double strands have been shown to stack in an end-to-end fashion to produce soft, liquid crystalline phases.[22,34] However, to our knowledge, this is the first report of fiber formation from short, blunt-ended DNA strands. These new 1D materials most likely contain highly aligned DNA duplexes in their core, and an oligoethylene glycol corona residing in an organic solvent. The hierarchical assembly of these fibers into ordered surfaces in the absence of divalent cations, and the inability of their single-stranded form to assemble into fibers, raise some mechanistic questions regarding their formation, which we are currently investigating. The ease of synthesis and assembly addressability of this new class of hybrids allows these molecules to lend their use in fields ranging from gene delivery vehicles, modular scaffolds for tissue growth,[35] addressable, dynamic tools for nanopatterning in 1D and 2D, and templates for nanowire fabrication.

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Supporting Information Available: Experimental methods, D-DNA synthesis, and additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(36) See the Supporting Information.
(37) Preliminary experiments on the effect of added divalent cations on the assembly of D-DNA **1** have been performed. Different concentrations of Mg^{2+} were added to the buffer/acetonitrile solutions, and the assembly was studied by AFM. At low Mg^{2+} concentrations (1 mM), abundant and bundled fibers were observed, possibly due to binding of Mg^{2+} and reduction in the DNA backbone electrostatic repulsion. However, at higher Mg^{2+} concentrations (4 mM), fibers were not observed. At this point, the D-DNA building blocks are likely coated with divalent cations, which may cause some charge inversion and as a result may affect the assembly energetics.