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Intercalators as Molecular Chaperones in DNA Self-Assembly

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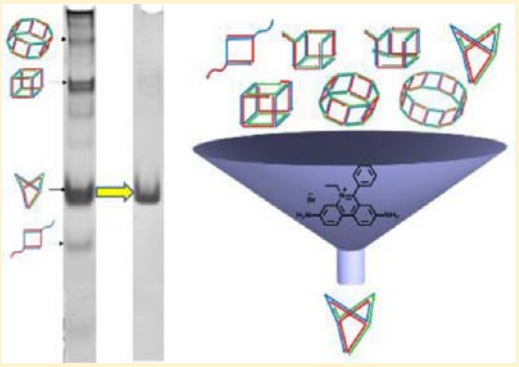
Supporting Information

ABSTRACT: DNA intercalation has found many diagnostic and therapeutic applications. Here, we propose the use of simple DNA intercalators, such as ethidium bromide, as tools to facilitate the error-free self-assembly of DNA nanostructures. We show that ethidium bromide can influence DNA self-assembly, decrease the formation of oligomeric side products, and cause libraries of multiple equilibrating structures to converge into a single product. Using a variety of 2D- and 3D-DNA systems, we demonstrate that intercalators present a powerful alternative for the adjustment of strand-end alignment, favor the formation of fully duplexed "closed" structures, and create an environment where the smallest, most stable structure is formed. A new 3D-DNA motif, the ninja star, was self-assembled in quantitative yield with this method. Moreover, ethidium bromide can be readily removed using isoamyl alcohol extractions combined with intercalator-specific spin columns, thereby yielding the desired ready-to-use DNA structure.



INTRODUCTION

DNA intercalation is a noncovalent interaction that results from the insertion of an aromatic molecule between the base pairs of double helical DNA. DNA intercalators have found multiple therapeutic and diagnostic applications, such as their use as antitumor and antibiotic reagents,[1,2] and as tools for DNA mismatch detection.[3,4] Intercalation imposes profound changes on DNA structure, such as stabilization of the DNA double-stranded form, unwinding, lengthening, and stiffening of the double helix.[5,6] Here, we examine how these properties can be used to aid in the error-free self-assembly of discrete DNA structures.

Despite its numerous successes,[7−9] DNA self-assembly is often complicated by the formation of misassembled oligomeric products, in addition to the desired structures. As the complexity of the final structure increases, multiple side products with dangling single-stranded ends can form, often significantly reducing the yield and purity of the desired product. We report the use of a common intercalator, ethidium bromide (EtBr), to influence DNA self-assembly, reduce the formation of oligomeric side products, and converge a library of multiple equilibrating structures into a single product. We have identified two factors that allow intercalators to guide DNA self-assembly: (i) the stabilization of double-stranded DNA (dsDNA) over single-stranded DNA (ssDNA), creating an environment where fully duplexed ("closed") structures are more favored than open structures; and (ii) a large change in DNA helical twist, which alters strand-end alignment and biases the product distribution to a new outcome. The resulting strand-end alignment can be predicted through simple modeling of DNA duplexes. Furthermore, we demonstrate that the intercalator used during assembly is easily removed using solvent extraction and a spin column.[10]

EXPERIMENTAL SECTION

For each assembly, equimolar amounts of each DNA complement were dried separately. Total single-stranded DNA content for each assembly was kept constant at 0.2 nmol. Each complement was resuspended in 1xTAEMg buffer (40 mM TRIS, 18 mM acetic acid, 2.5 mM EDTA, 12.5 mM $MgCl_2$) such that the total volume of all of the complements was 10 μL. Complements were then vortexed thoroughly, followed by brief centrifugation. 2 μL of intercalator solution were gently added to one complement. Each of the remaining complements was then added to the one containing intercalator solution. No mixing was used at this stage (see the Supporting Information). The combined sample was then treated to an annealing protocol consisting of a ramp to 95 °C followed by a 3.5 h cooling to 4 °C. Samples were stored at 4 °C until analyzed.

RESULTS AND DISCUSSION

To test the ability of intercalators to guide DNA assembly, we deliberately designed a three-dimensional DNA system that can come together into a number of possible structures. This system is formed of three simple, unmodified DNA strands. Strands **1** and **2** possess two complementary 10-mer sequence stretches (**1** has A,B and **2** has A′,B′; A is complementary to A′ and B to B′), flanked by repeating 10-mer stretches C (see Scheme 1a). Strand **3** possesses four C′ stretches, each complementary to C. Each sequence stretch in strands **1**, **2**, **3** is separated from the next by two thymidine spacers.

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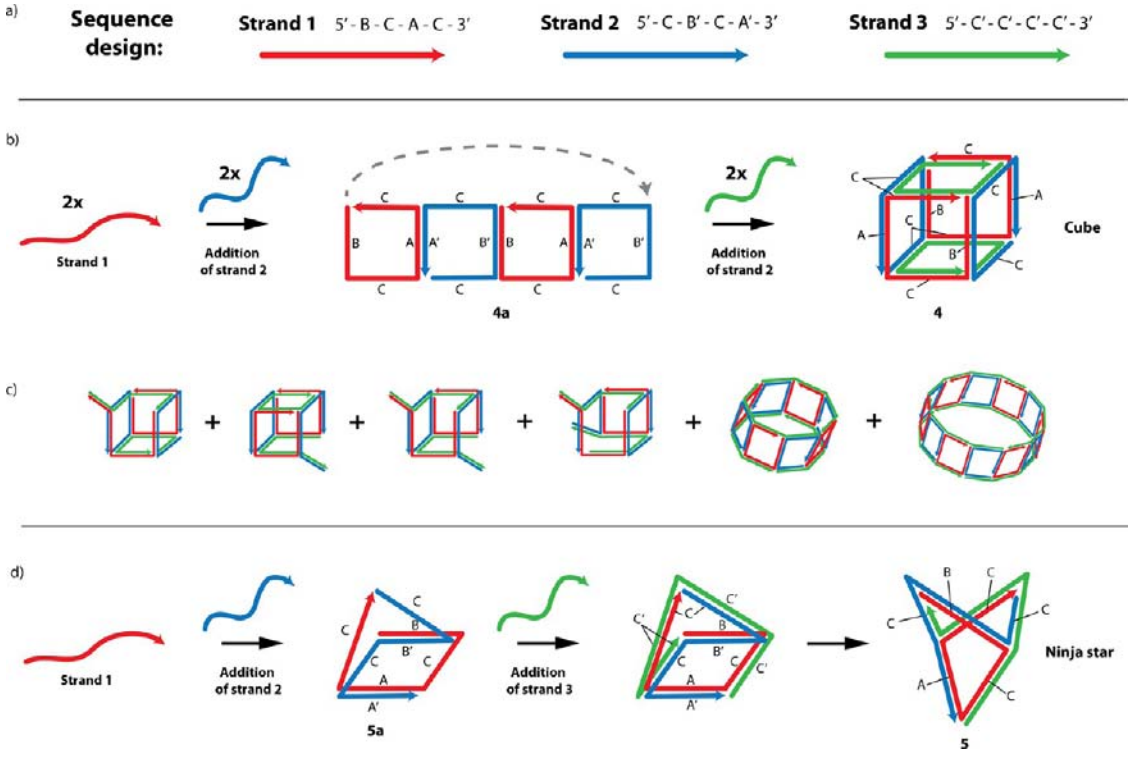
Scheme 1. (a) Sequence Design of the 3 Strands; (b) Assembly of Cube 4; (c) Possible Open Cube and Higher-Order Products; and (d) Assembly of Ninja Star 5.



Because of their sequence symmetry, there are two ways in which strands **1** and **2** can hybridize together: one possibility is that they align their complementary ends in a side-by-side arrangement, to form intermediate **4a**, as shown in Scheme 1b. Two strands **3** can then hybridize to the four aligned C stretches on the top and bottom of **4a**, to form a cubic structure **4** (with a 2:2:2 ratio of the strands, see Scheme 1b).

A second possibility is that strands **1** and **2** cyclize together to hybridize both pairs of complementary strands A:A′ and B:B′ and form intermediate **5a** (Scheme 1d). If strand **3** were then to fully hybridize to the C stretches of **5a**, the product would be an unusual propeller-shaped molecule **5**, which we term ninja star (with a 1:1:1 ratio of the strands, Scheme 1d). If we follow each strand in this structure, we see an S-shaped pattern in how it comes together with its complementary strands. We believe that the ninja star is the first DNA structure reported to follow this S-shaped pattern.

Note that many other "open" cubes can result, by shifting the hybridization of strands **3** along the top and bottom of intermediate **4a** (Scheme 1c). Higher-order structures can also include octamers and dodecamers, in addition to open oligomeric structures with dangling ends (Scheme 1c).

Strands **1**, **2**, and **3** were annealed, followed by analysis by gel electrophoresis under native conditions (Figure 1). This revealed the formation of a mixture of products, with the ninja star making up 24% of the final products and cube molecules 8%, along with a number of most-likely open intermediate structures (37%), and a significant amount of oligomers (17%). We then annealed the three strands in the presence of increasing amounts of ethidium bromide. Interestingly, the entire library of products converged to the ninja star structure, with no oligomers or open products detected at high intercalator concentration. Band assignment of these structures was confirmed by sequential assembly under

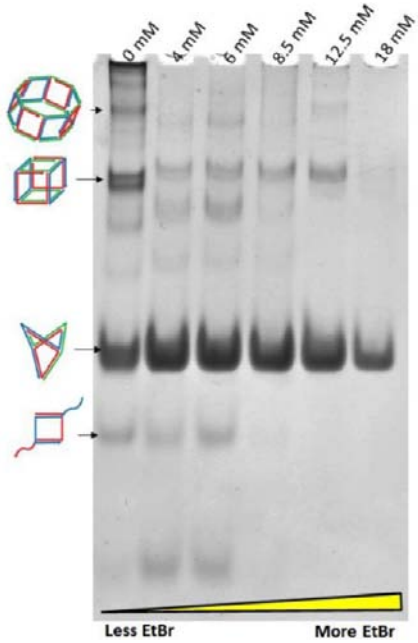


Figure 1. Assembly of strands **1**, **2**, and **3** in the presence of increasing amounts of EtBr (concentration of base pairs = 1 mM). The concentrations of the 2 μL EtBr solutions are indicated above each lane.

different conditions and exonuclease digestion experiments (see the Supporting Information). Thus, by adding a common intercalator, we were able to take a system with numerous products, and reduce it to the single smallest structure containing fully duplexed DNA. This demonstrates that the intercalator EtBr biases a library of structures not only toward

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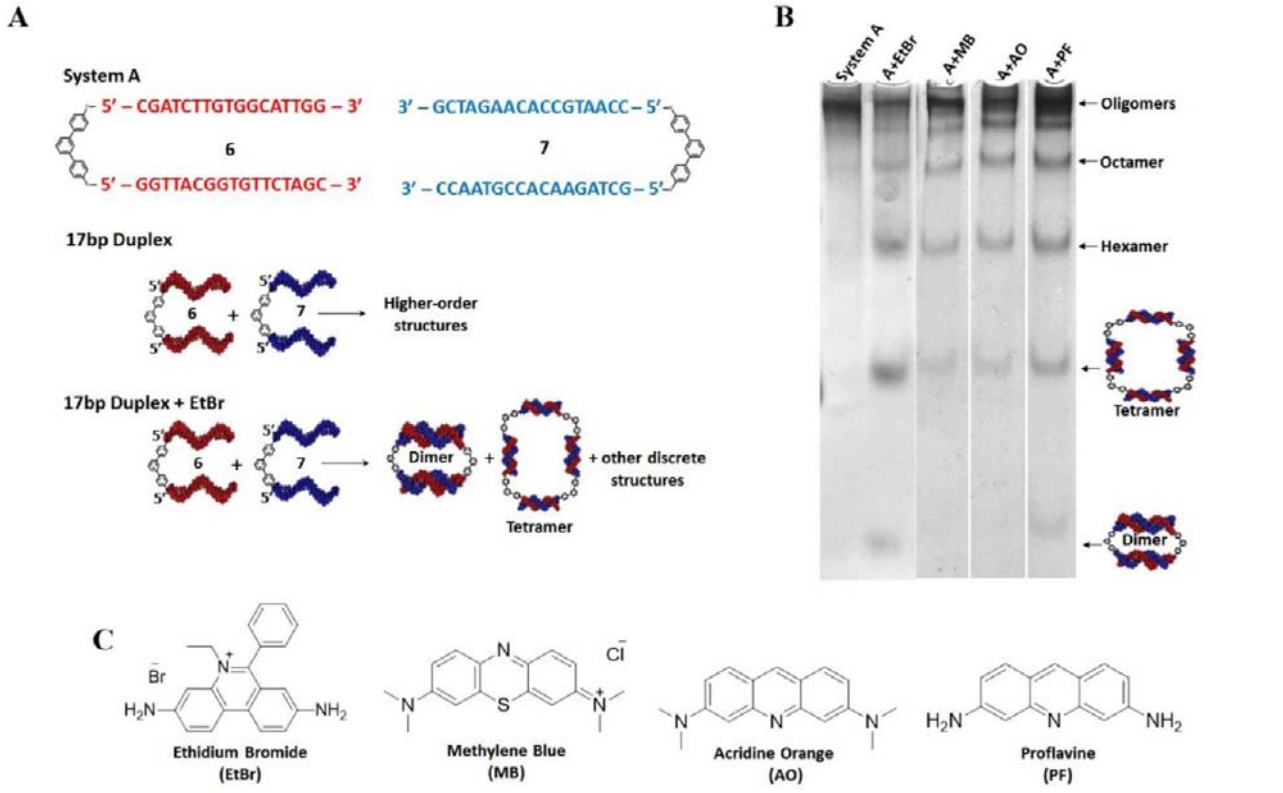

Figure 2. (A) Sequence and connectivities of strands **6** and **7**. (B) Assembly of strands **6** and **7** in the presence of each intercalator. (C) Structures of four intercalators.

closed structures, but also chaperones the formation of a specific closed structure (ninja star) over other possible structures (cube and higher-order structures).

We were interested in further examining the mechanism by which this selection occurs. Intercalators can have a dramatic thermodynamic effect on DNA structure, such as stabilization of DNA duplexes, significant unwinding, lengthening, and stiffening the double helix, and introducing more sterically demanding substituents to the DNA base stack. Less well explored is their kinetic effect on the DNA self-assembly process and their potential to accelerate the formation of some structures over others.

We thus turned our attention to a simpler, two-dimensional DNA assembly. We had previously reported a system in which two components **6** and **7** contain complementary 17-base stretches that are bridged on their 5′-ends by a short triphenylene spacer (Figure 2A). Because of the size and rigidity of the organic linker and the difficulty in aligning both strand ends to form discrete structures such as dimers or tetramers, this structure was previously shown to only result in open, higher-order oligomers.[11] As a preliminary screen for the chaperone effect, several molecules known to intercalate with DNA were chosen: ethidium bromide, methylene blue, acridine orange, and proflavine (Figure 2).[12] Experimental results are given in Figure 2B. The results show a significant increase in the assembly of discrete products, and a corresponding decrease in higher molecular weight oligomers upon addition of intercalator. This again indicates that the presence of the intercalator has a significant impact on the product distribution. For further studies, EtBr was chosen due to the wealth of available literature on its interaction with DNA.

We were interested in monitoring this self-assembly process with increasing amounts of ethidium bromide solution. Strands **6** and **7** were annealed with five different EtBr solutions ranging from 0 to 17 mM (Figure 3). The addition of even a small

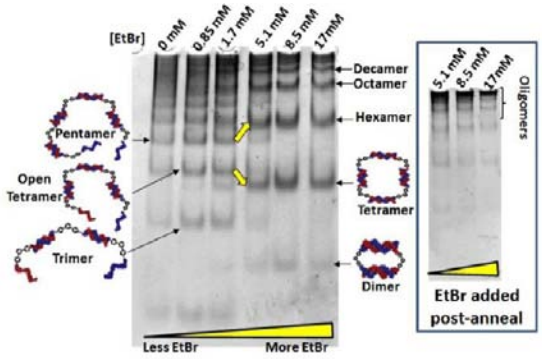

Figure 3. Strands **6** and **7** annealed in the presence of increasing amounts of EtBr. Yellow arrows indicate conversion of the open structures to cyclic structures with addition or EtBr. Inset: The same system with EtBr added after the anneal step.

amount of EtBr to this system prior to annealing results in the formation of a number of discrete structures, including both higher-order cyclic structures and lower-order open structures (see the Supporting Information for characterization of open and closed structures). When [EtBr] = 1.7 mM (an EtBr:base pair ratio of 1:1), bands corresponding to lower-order closed structures begin to appear. As [EtBr] is increased from 1.7 to 5.1 mM, the closed structures become more prominent (marked by the yellow arrows). At [EtBr] = 8.5 mM (ratio

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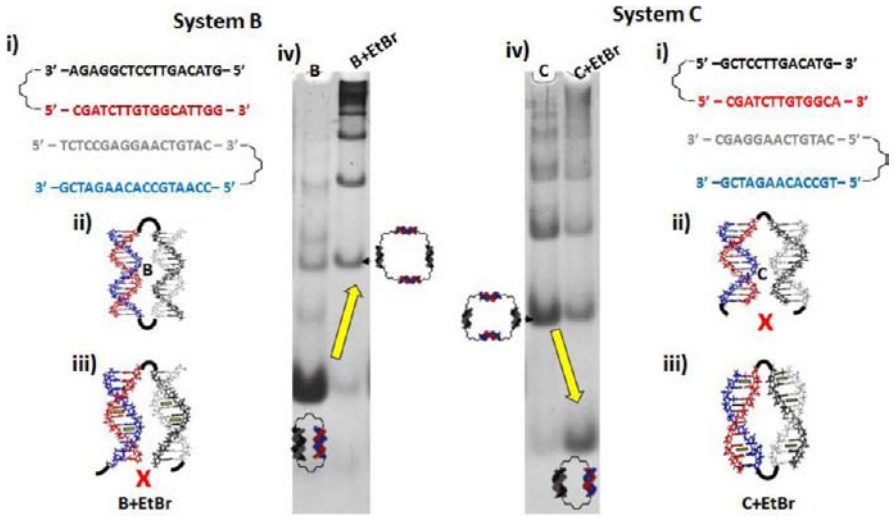

Figure 4. Systems B (left) and C (right). (i) Sequences and connectivities. (ii) Strand-end alignment models for assembly without intercalators. A red "X" indicates unfavorable alignments. (iii) Strand-end alignment for duplexes corresponding to the helical twist of each system annealed with EtBr. Intercalators are roughly represented as boxes within the duplexes. (iv) Assembly gels for systems annealed in both the presence and the absence of EtBr.

of 5:1), no open structures remain, and all structures are fully hybridized cycles. This indicates that the addition of EtBr changes the structural distribution, as well as induces the cyclization of the DNA strands. Thus, the addition of DNA intercalators results in the hybridization of dangling strand-ends into double-stranded closed structures. We also ascertained that the addition of EtBr was necessary during the annealing step. A series of controls in which samples were annealed prior to the addition of EtBr showed little re-equilibration of the product distribution (Figure 3, inset). This indicates that EtBr is chaperoning assembly instead of influencing preassembled products, a result that is consistent with the observations in the three-dimensional system above.

In addition to forming closed, fully hybridized structures, we also noted that the tetramer is more prevalent than the dimer, even though it is composed of a greater number of molecules. This is true not only for the system A mentioned earlier (which normally forms oligomers), but for a second system (B), which we had previously shown to favor dimer self-assembly.[11] This system also uses 17mers, but they are linked in a 5′-3′ fashion by a flexible hexane diol linker. When annealed with EtBr, we see a marked decrease in dimer formation and an increase in the formation of tetramer, hexamer, and other higher-order structures (Figure 4).

The preference for tetramer over dimer can be due to two possible structural effects of the intercalator: (i) intercalation introduces additional steric requirements into the DNA strands (for example, the phenyl substituent of the ethidium bromide, now oriented into the DNA grooves). The dimer structure features closely packed DNA duplexes (Figure 2A), and may be more difficult to form with this new steric requirement. (ii) Intercalation unwinds the DNA duplex, changing the helical twist of DNA and thus aligning the strand ends into a different orientation, which favors closure of a different DNA cycle.

We explored the first possibility by using a different system, which we have previously shown to favor tetramer over dimer (system C, Figure 4). This system uses the flexible linker hexane diol; its linked strands are only 13 bp long, and they are both connected to the linker via their 5′ ends. In this system, tetramer is a preferred product because the strand ends cannot align to close a dimer (Figure 4ii, right). If the dominant effect of ethidium bromide is to increase the steric requirements around each DNA duplex, then the self-assembly in this second system should not change in outcome, as it already produces the tetramer with strands further apart than in a dimer structure. However, as can be seen in Figure 4, right, this is not the case: instead, there is a shift toward dimer formation. Thus, steric effects introduced by the intercalation of many ethidium bromide molecules are most likely not the dominant effect in this system.

The second possibility is that the intercalator changes the structural requirements through a change in DNA helical twist. We have shown previously that adjusting the strand-end alignment in a system can modify the overall structural distribution, especially for duplexes connected by rigid linkers. Previously, we altered strand-end alignment by either changing the connectivity between the linker and the DNA strands or changing the total number of base pairs in the system. Recently, Shih and co-workers[13] demonstrated that insertion of intercalators into DNA duplexes has a demonstrable effect on the helical twist of a duplex and the assembly of DNA origami structures.

For a natural B-DNA duplex, the helical twist has been measured at 36° per base pair.[13,14] The total helical twist (H) for a 17 bp strand can then be calculated as $H = 36 \times 17 = 612°$. Intercalation of ethidium bromide is known to unwind DNA and reduce the DNA helical twist by ∼26°/intercalation event.[14] Moreover, due to the neighbor exclusion principle, only about 1 in 2 interbase pair sites are available for binding the intercalator.[6,15,16] A total of eight intercalators can be accommodated in a 17 bp duplex (see Figure 5B). Thus, a 17 bp duplex with 8 intercalators can have a total helical twist $H_i = 612° - (26° \times 8) = 404°$ (Figure 5B). As such, rather than a reciprocal twist density of 10.5 bp/turn for a regular B-DNA, a fully intercalated duplex with EtBr will have a reciprocal twist density of ∼15 bp/turn. Therefore, a fully intercalated 17 bp duplex has a helical twist similar to an ∼11 bp regular B-DNA duplex. A simple model of system A shows that a dimer from such duplexes is unable to form due to unfavorable strand-end alignment, while tetramer formation is more favorable (see

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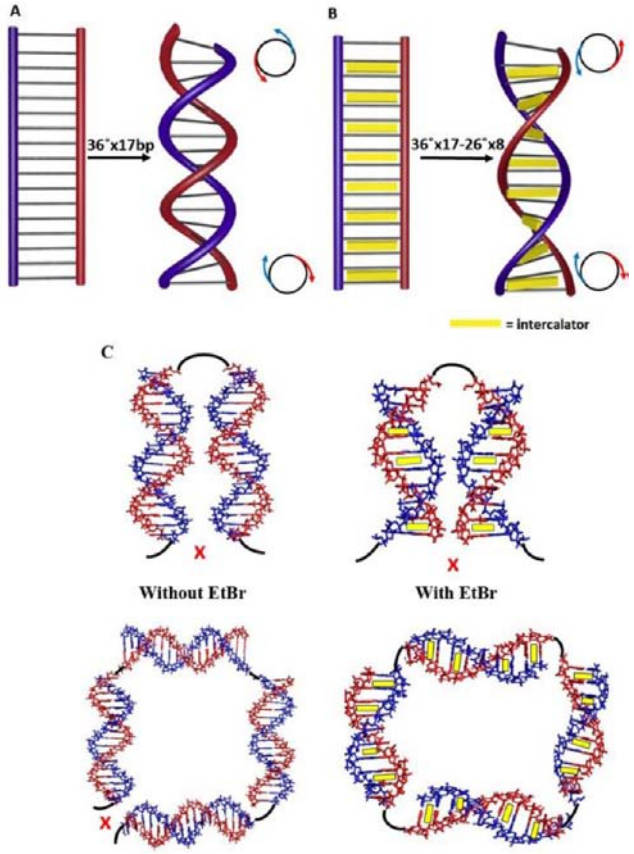

Figure 5. (A) A 17 bp duplex has a helical twist of 36°. (B) A 17 bp duplex with intercalators inserted in every other interbase pair space has a smaller helical twist of (36° × 17) − (26° × 8) = 404°. (C) Models showing how strand end alignment affects dimer and tetramer formation.

Figure 5C and the Supporting Information). This is consistent with the observation that ethidium bromide biases the library of products away from dimer formation. On the other hand, a 13 bp intercalated system (Figure 4, right) has a helical twist similar to a ∼9 bp regular DNA strand. Now the strand-end alignment is favorable for dimer formation, consistent with the PAGE results. From these experiments, it can be concluded that strand-end alignment is a significant factor in determining product outcome.

To gain more insight into the re-equilibration process as a function of time, EtBr-treated samples were rapidly heated, then allowed to cool at three different rates, from very fast quenching (1−2 min) to slow cooling (3.5 h). These results were compared to nonintercalator treated samples at the three different cooling rates (Figure 6).

We were able to make two deductions from this experiment. First, the preference for closed, double-stranded structures in samples annealed with EtBr arises even at the early stages of the annealing process. Second, the product distribution changes as the cooling rate is decreased, with disappearance of the strained dimer in favor of the tetramer and higher-order cyclic structures with better strand-end alignment. Thus, there may be two stages of selection with this system: first, closure of open structures to all-duplex cycles, followed by equilibration in favor of the least strained (tetramer) product.

The above experiments again illustrate the ability of DNA intercalators to favor the formation of closed structures over

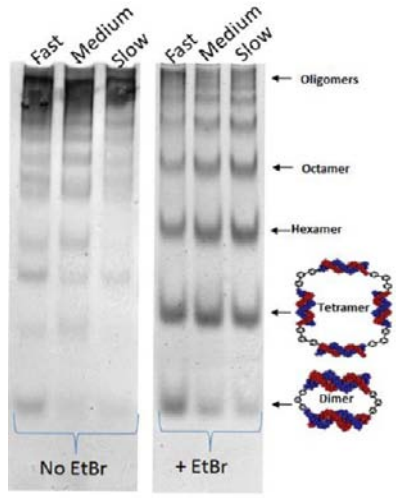

Figure 6. Strands **6** and **7** annealed at three different speeds. (Left) Without EtBr. (Right) In the presence of EtBr.

open-ended oligomeric structures, which can be a powerful "error-correction" tool in DNA nanotechnology.

Finally, we were interested in probing the generality of this EtBr-chaperoned re-equilibration. For this, we chose a literature-based DNA tetrahedron[17] (system T) that has been adapted for many different applications[18−22] and studied its self-assembly in the presence of a 12.75 mM EtBr solution (5:1 EtBr:base pairs, Figure 7).

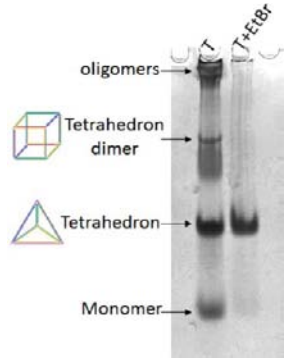

Figure 7. Assembly of a tetrahedron in the absence and presence of EtBr.

Similar to the 3D-ninja star system, use of EtBr during the annealing process results in the formation of the desired product (tetrahedron) in near-quantitative yield, whereas annealing without EtBr gives a range of products (this was tested both for the conditions defined in previous reports[18] and under the same buffer/anneal conditions used in this work). This again is strong evidence that the effect of intercalators on DNA assembly is generally applicable.

This method of intercalator cleanup gives excellent yields and consistent results. However, the presence of intercalator in a sample may limit the possibilities for its future use. As such, we developed a protocol for extracting the intercalator without disturbing the final assembly products. Several methods of intercalator extraction were tested, including extraction with a variety of purification columns (see the Supporting Information) and solvent extraction.[10] The most effective method involved increasing the salt concentration, followed by

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extraction with isoamyl alcohol and use of an EtBr Minus spin column (Sigma Aldrich). The extraction efficiency was determined using a combination of techniques, including circular dichroism, UV/vis, gel electrophoresis, and fluorescence (see the Supporting Information). As a final determination for EtBr removal, a fetal bovine serum (FBS) assay was performed. FBS is a mixture of enzymes widely used to culture eukaryotic cells in vitro, among which DNA degrading nucleases can be found. EtBr is known to deactivate many of these enzymes, resulting in less DNA digestion (see the Supporting Information for methods).

As evidenced by Figure 8, enzyme digestion of samples extracted using the combined method is indistinguishable from

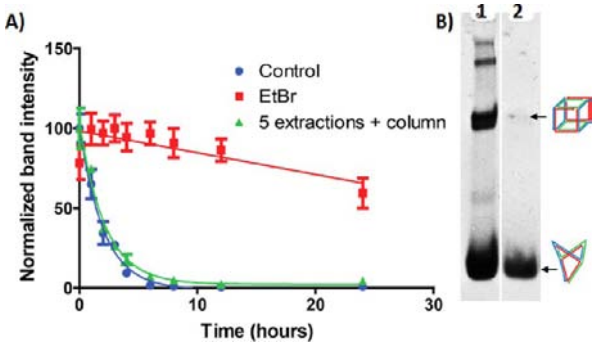

Figure 8. (A) FBS assay of the ninja star system. Blue "●" represent DNA annealed without EtBr. Red "■" represent DNA annealed in the presence of EtBr but not extracted. Green "▲" represent sample annealed in the presence of EtBr and extracted using a combination of increased salt concentration, liquid extraction, and spin columns. (B) Lane 1 shows product distribution of the ninja star system without the addition of EtBr; lane 2 shows the product distribution following use of EtBr and extraction.

the samples that were never exposed to EtBr, indicating that no residual EtBr remained after extraction (95% confidence interval, see the Supporting Information). Furthermore, gel analysis reveals that the structure isolated during EtBr cleanup remains assembled through the extraction process (Figure 8B). These results, along with those presented in the Supporting Information, strongly indicate that structures assembled using an EtBr chaperone can be easily recovered for future use.

■ CONCLUSIONS

The analysis of the effects of an intercalator, specifically EtBr, on DNA assembly has resulted in a remarkable, easy-to-use tool capable of cleaning up and re-equilibrating DNA assemblies. We identified two main factors that influence how this intercalator guides structural assembly. First is the tendency for formation of closed structures with duplexed rather than single-stranded DNA ends. Second is the strand-end alignment, in which the intercalator significantly reduces the DNA helical twist and biases the product distribution to a new outcome. With these new criteria, the addition of intercalator can result in the full assembly of the smallest, most stable, fully duplexed product. A new 3D-DNA motif, the ninja star, was self-assembled in quantitative yield using this method. Furthermore, the intercalator can be readily removed from the assembled products through a combination of solvent extraction and spin columns.

■ ASSOCIATED CONTENT

Supporting Information

DNA system sequences, assembly and characterization, mixing methodology, modeling, additional control experiments, and extraction studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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