Competitive annealing of multiple DNA origami: formation of chimeric origami

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Abstract
Scaffolded DNA origami are a robust tool for building discrete nanoscale objects at high yield. This strategy ensures, in the design process, that the desired nanostructure is the minimum free energy state for the designed set of DNA sequences. Despite aiming for the minimum free energy structure, the folding process which leads to that conformation is difficult to characterize, although it has been the subject of much research. In order to shed light on the molecular folding pathways, this study intentionally frustrates the folding process of these systems by simultaneously annealing the staple pools for multiple target or parent origami structures, forcing competition. A surprising result of these competitive, simultaneous anneals is the formation of chimeric DNA origami which inherit structural regions from both parent origami. By comparing the regions inherited from the parent origami, relative stability of substructures were compared. This allowed examination of the folding process with typical characterization techniques and materials. Anneal curves were then used as a means to rapidly generate a phase diagram of anticipated behavior as a function of staple excess and parent staple ratio. This initial study shows that competitive anneals provide an exciting way to create diverse new nanostructures and may be used to examine the relative stability of various structural motifs.

The commercial development of inexpensive and quickly produced DNA of arbitrary nucleobase sequence has fueled the growth of DNA nanotechnology as a field [1]. DNA nanotechnology has made promising advances toward light harvesting [2], computation [3], cancer treatment [4, 5], and assembly of nanoelectronics [6]. While many DNA self-assembly strategies exist [7, 8], scaffolded DNA origami has received significant attention as a convenient way to design and create discrete nanoscale objects [9]. Scaffolded DNA origami consist of single strand DNA (ssDNA) of two types, synthetic oligomers and circular viral genomes; the viral scaffold is forced to route through the designed structure by the binding of complementary subsequences on the synthetic oligomers, or staples. Staples are added in excess, often 10× relative to the scaffold concentration, strongly driving the viral ssDNA scaffold to fold into the target structure. Although 10× is a common staple excess anneals have been successfully performed as low as 2.5× staple excess. As this study involves multiple staple pools, staple excess will always refer to the total staple concentration relative to the scaffold, while the parent staple ratio will refer to how much of that total corresponds to the staple pool for each target origami. High production yield is achieved by thermal annealing and slow cooling of the system. Such annealing has also been performed chemically, isothermally, and mechanically [10–12].

The hybridization of double strand DNA (dsDNA) has been well studied, particularly under physiological conditions [13, 14]. The formation of dsDNA from ssDNA is driven by base stacking interactions and is highly influenced by the GC content of the nucleobase sequence, the sequence length, and the salt concentration [13]. The energetics of these systems are predicted well by the nearest neighbor model, which accounts for sequence and length, and can be corrected for salt concentration [15, 16]. Often, the thermodynamics of DNA systems are...
discussed in the context of the melting temperature, or \( T_m \), which is defined as the temperature at which half of the dsDNA has been denatured and dissociated into ssDNA.

The formation of DNA origami is much more complex than the hybridization of two ssDNA strands, and cannot be accurately predicted with only the nearest neighbor model [17, 18]. The 64–200 synthetic staple strands can have three or more subsections which bind to different positions along the viral scaffold (typically separated by Holliday junction-like crossovers in the target structure), and each subsection can form dsDNA independently of the others. Each of these segments can exhibit cooperative behavior with its neighbors, which is to say that each binding will make the binding of other nearby staples more favorable [18–20]. The cooperativity in DNA origami systems results in hysteresis between melt and anneal curves [20, 21]. The annealing temperature, \( T_A \), can be different from the \( T_m \) for an origami system. As this study addresses simultaneous competitive annealing, \( T_A \) will be used rather than \( T_m \). The cooperativity comes from the added stability of base stacking with neighboring dsDNA domains, and conformational entropy penalties of folding the large scaffold strand. As viral scaffold is circular, entropy is reduced as the main loop shrinks and as it is divided into smaller loops. Staples which bridge disparate scaffold sections which are initially unstable due to conformational entropy penalties will see that penalty decrease as other nearby staples bind the scaffold. The number of possible binding events, the huge number of possible orderings of those events, and their cooperative interactions, complicates the relationships between origami design and folding.

Additionally, characterizing the formation of DNA origami is more complex than for hybridization of two ssDNA strands with complementary nucleobase sequences. Techniques which are sensitive to the amount of ssDNA or dsDNA present, such as UV–vis absorption spectroscopy or fluorescence spectroscopy with an intercalating dye, cannot determine which strands are hybridizing [13, 22, 23]. Strand specific techniques can give extensive detail, but require expensive chemical modifications to many ssDNA strands [21]. Techniques typically used to image DNA origami (e.g. AFM) are difficult to perform in liquid at high temperature, and require surface binding which drastically changes the energetics of formation [24]. Understanding DNA origami folding pathways has been a major point of inquiry for DNA nanotechnology, and has been the subject of numerous studies [10, 19, 21, 25].

Of particular relevance to this study is the recent development of dimer scaffold systems and surface quenching [19, 25]. Dunn et al developed a dimer origami scaffold, in which the 7.2 kb M13 scaffold nucleobase sequence is repeated twice in a single, 14.4 kb molecule [19]. This system ensured that whenever a subsection of a staple bound, its other subsections had to choose between two binding locations with perfectly complementary sequences. The nearest neighbor model predicts identical energetics for these options, so binding preferences result from sources not considered in that model. The resulting origami consisted of two copies of the tall rectangle origami with a discrete connecting edge/point. The distribution of connections was nonrandom; the choices made during initial binding which pushed the dimer along particular folding pathways. This distribution was used to determine the preferences in those staple binding events; which encouraged even spreading of the conformational entropy penalties across as many of the binding events as possible. This preference is supported in a recent study characterizing the folding process by using surface binding to quench, or arrest, the folding process at intermediate states [25].

In this study we intentionally frustrated the folding process by forcing competition for the scaffold strand between the staples for different origami structures. To achieve this a similar experimental approach to the dimer scaffold was used; rather than a single staple set with a dimer scaffold, a single scaffold was thermally annealed with mixed pools containing full staple sets for different parent origami, as illustrated in figure 1. An important distinction between these strategies is that the binding of any staples from either structure will shift the relative staple concentration of the parent origami, resulting in a more dynamic system. Also, while the dimer scaffold consisted of two repeats of the same sequence, the parent staple pools are distinct both in structure and in sequence. This should result in much more pronounced frustration of the folding process than with the use of a dimer scaffold.

Such a competitive, frustrated anneal will be an even more complex process than the folding of a typical origami nanostructure. Three results of such systems are readily anticipated. The first being that the frustrated folding results in partially unsatisfied staples which then bind to additional scaffold molecules, ultimately polymerizing into aggregates. The second outcome is the complete dominance of one parent structure, due to a generally higher \( T_A \) or to strong cooperativity. Finally, and most interestingly, is the possibility that the resulting nanostructure will inherit regions from each of the parent origami designs. In all cases, the ability of DNA strands which share parts of their nucleobase sequences to dynamically displace one another should play an important role [26]. This strand displacement should resolve the frustrated folding, as cooperative effects encourage substructures to nucleate, grow and compete with one another. More detailed thought experiments are included in section 1 of the supplementary data.

This study shows that competitive, simultaneous anneals can create chimeric nanostructures inheriting parts or regions of both parent origami structures. Given the resource investment associated with quantifying
chimera formation using AFM, initial surveys using fluorescence spectroscopy anneal curves were used to map conditions where interesting chimeras might form. Information from these surveys was then compiled into a phase diagram. Such phase diagrams could be particularly useful as they may provide a prediction of what structures will form during a competitive anneal, at minimal time costs.

Knowledge gained from competitive annealing could be leveraged to design origami whose chimera are predetermined and dependent on annealing conditions, further improving the robust structural diversity of DNA nanotechnology. An example of such a strategy would be to design a system in which the kinetic pathway and the thermodynamic free-energy minimum conformation are different, resulting in chimeras which inherit different regions of the parent origami depending on cooling rate. As techniques to probe the formation of DNA nanostructures improve, so does our ability to create complex, and potentially revolutionary new tools [12, 18, 19, 25].

Results and discussion

Competitive anneals and chimeric origami

As a first step, this study sought to form and analyze chimeric origami which inherit structural regions from both parent origami. It is important to compare the two designs used, shown in figures 2(A) and (B) and described in the methods. Figure 2(A) shows the routing map of the 2D circle and 3D six-helix bundle miniM13 origami [27]. The routing map shows only the scaffold, and has been colored in 400 base increments along the 2.4 Kb scaffold length. Regions of each origami of corresponding color represent the same sequence and section of the scaffold.

Figure 2(B) shows the circle maps of both miniM13 origami. The outside ring of the circle map represents the scaffold sequence starting from the bottom center. The arcs in the circle map illustrate the distance along the scaffold that an individual crossover brings together. Such a map is valuable as a larger arc indicates a larger conformational entropy penalty for that crossover, if it were the first to bind. It should be noted that as the structure folds, these penalties will change, so the circle map cannot be used directly to predict T\text{\textit{m}}/T\text{\textit{A}} or other properties. By comparing these circle maps two valuable pieces of information can be found. First, the bundle has many more crossovers bridging distant regions of scaffold. Second, there are some sections of the scaffold which fold over short distances for each parent structure. In the bundle this is the red section, while in the circle origami it is the very top (yellow/orange) and very bottom (green/black) (see figure 2(A)). Figure 2(C), shows the T\textsc{\textit{s}} for both the circle and bundle as a function of staple excess relative to the scaffold. The circle, when annealed at the same staple excess as the bundle, consistently reaches 50% hybridization at a higher temperature.

To maximize the probability of observing chimeric nanostructures, initial systems were annealed at high scaffold concentration, 25 and 50 nM scaffold, and at a relatively low staple excesses (5×) at a cooling rate of 0.125 °C min\textsuperscript{-1}. For both concentrations three samples were annealed with varying parent staple ratios. The bundle staples consisted of 33%, 50%, and 66% of the total staple concentration for these anneals. AFM images of the 25 nM samples are shown in figure 2(D), images of 50 nM scaffold concentration are shown in section 2 of the supplementary data.

In the competitive anneals figure 2(D), chimeric nanostructures are easily observed. It is also evident that both the number and types of structures vary with the ratio between staple species. In each of the competitive anneals there are also origami aggregates. For the purpose of this study, we will consider discrete multiscaffold
chimeric origami to be different from the aggregates, in which many scaffolds appear to have polymerized into a large mass which cannot be readily characterized. It is likely that the formation of origami aggregates in figure 2 were driven by the high scaffold concentration and the low total staple concentration.

Qualitative analysis of single scaffold chimera
To better understand the types and prevalence of chimeric structures, new samples were annealed with a higher total staple excess \((7.5 \times)\) to minimize the probability of polymerization. To examine if kinetics were a determining factor in chimera formation a sample grid was created varying the parent staple ratio, anneal rate, and scaffold concentration. Each was tested at three values, resulting in 27 samples. Concentration consisted of 5 nM, 15 nM, and 25 nM scaffold. Annealing rates examined include 0.0625, 0.15, and 0.58 \(^\circ\)C Min\(^{-1}\). The staple content consisted of 33%, 50%, and 66% bundle staples.

During image analysis (described in methods) example chimera images were noted, saved and set aside whenever a visibly different chimera was seen regardless of which anneal the sample came from. These images were compiled to illustrate the very broad range of chimeric morphologies observed for single scaffold origami (figure 3(A)). Based on this taxonomy, it appears that all observed single scaffold chimera inherit the top and bottom portions of the circle and the middle of the bundle. These locations correspond to the regions with short arcs in the circle maps shown in figure 2(B). The AFM image in figure 3(B), which contains a full circle origami and a chimeric origami, sheds further light on this preference. Direct observation of the helices in the circle portions of the chimera identifies the scaffold region folded into bundle (light green/red).

From the taxonomy of single scaffold chimeras in figure 3(A) and from figure 3(B), we propose a routing map and folding pathway for the single scaffold chimera, shown in figure 3(C). This path is supported by the taxonomy in figure 3(A), as the distribution of shapes could be explained as the nucleation of bundle and circle origami at their respective locations followed by growth. The dotted lines in the center of figure 3(C) indicate anticipated cooperative pressure of the nucleated regions. The growth of the single scaffold chimera would be determined by this pressure, as modulated by the parent staple ratio. The routing pattern shown in figure 3(C) would also effectively explain why the single scaffold chimeras appear to group in discrete bundle lengths rather than displaying a uniform sampling of the possible lengths. It may be that chimeras are only stable when the transition between scaffold routing patterns of the circle and bundle has a minimal gap in which neither structure is satisfied, so the bundle can likely only grow in lengths corresponding to two helices of the circle architecture.
The proposed routing pattern shown in figure 3(C) is also consistent with observations of the role of conformational entropy in DNA origami folding. Sections of the circle and bundle which appear to nucleate the parent structures correspond with sections on the scaffold which have a low initial conformational entropy penalty in one parent structure and a high penalty in the other. As shown in figure 2(B), bases 1200–1600 of the miniM13 scaffold, colored red, have a high conformational entropy penalty indicated by a large crossover arc in the map for the circle origami, but relatively low penalties in the bundle. The opposite is true for the top and bottom sections of the circle, and their competing positions in the bundle.

Multiscaffold chimeras (figure 4) formed fairly rarely under these conditions. Unlike in the initial samples containing high scaffold concentration and low staple excess, no large scale polymerization was observed. These multiscaffold chimeras contained a discrete number of scaffold molecules, usually two. While none of the images were of sufficient quality to directly infer scaffold routing by helix counting, a preliminary routing pattern is proposed (figure 4) based on the pattern from figure 3(D). Given the symmetry in the observed multiscaffold chimeras and lack of free scaffold in the images, it is likely that the two scaffold molecules meet within the ‘body’ of the ‘double necked guitar’. Through this qualitative image analysis, we have examined the
range of chimera origami observed across a broad set of annealing and system conditions. While the set of samples here does not exhaustively test all possible conditions, it is sufficient to indicate that there is a broad range of processing conditions under which chimeric origami can form.

Quantitative analysis of single scaffold chimera

Qualitative examination of AFM images provides useful insights into how the chimeric structures form, into why certain regions may be more likely to be inherited, and into the breadth of processing conditions which form chimera. However, more rigorous analysis is required to understand the role that scaffold concentration, parent staple ratios, and annealing rate play in chimera formation. Using the technique detailed in the methods section, the total fraction of scaffold folding with circle staples was calculated and plotted as a function of scaffold concentration (figure 5A).

Not surprisingly the ratio of parent origami staples is the dominant factor in how bundle-like or circle-like an anneal will be, as shown in figure 5(A) (additional data is provided in the supplementary excel file). Circle is preferentially formed with circle-rich staple ratio (red lines), the fraction of bundle formed increases in evenly divided (purple lines), and bundle-rich staple ratios (blue lines), however the fraction folding to circle always exceeds the staple ratio. Comparatively, concentration, as plotted on the x-axis, does not appear to play a significant or consistent role over the range of concentrations tested. Surprisingly, given that concentration does not appear to have a strong impact, changing the annealing rate does affect the chimera formation. As the anneal rate goes from fastest rate (square-dotted line), to moderate rate (circle-dotted line), to the slowest rate (triangle-filled line), the overall fraction of scaffold folding into circle decreases. That a slower anneal rate results in a more bundle-like system suggests that the samples are not being cooled in equilibrium conditions; the necessity of strand displacement is likely to significantly increase the timescale required for the samples, particularly the 3D bundle, to reach equilibrium. Additionally, one would anticipate that the bundle, being a 3D shape, would have a more kinetically frustrated folding process than the 2D circle, so it is not surprising that faster annealing rates would favor the circle. It is worth noting that the bundle is not composed of enough helicities for staples to have much difficulty accessing the center of the structure; rather, the topological complexity associated with
crossovers bridging helices in multiple directions could cause frustration. It is then possible that the kinetically trapped regions would form circle rather than create a misfold.

To gather additional information on the complex folding processes of these competitive systems, we collected annealing curves using intercalating dyes (as described in the Methods section). Since intercalating dye fluorescence is unable to distinguish formation of one structure from the other, we chose to examine the anneal curves of the parent or target structures independently of each other, rather than attempt to deconvolute a competitive anneal. Therefore, two fluorescent anneal curves at 5 nM scaffold (one with 3.75 × circle staples and another with 3.75 × bundle staples) were compared to the AFM results for a competitive 5 nM scaffold anneal at 7.5 × total staple excess split 50/50 between the circle and bundle staple sets. This approach has the advantage that an anneal curve may be used to provide insight into several potential competitive conditions. For example, the anneal curve for 2.5 × circle staples may be compared to the anneal curve for 2.5 × bundle staples or for 5 × bundle staples allowing respective insight into a competitive anneal at 5 × total staple excess (at a 50-50 circle-bundle target staple ratio) or a competitive anneal at 7.5 × total staple excess (at a 25-75 circle-bundle target staple ratio), respectively.

In order to compare AFM data for competitive anneals to the separate anneal curves for each of the target origami one must assume simple competitive behavior within the system; that each staple of each structure anneals at a similar temperature to its competitor from the other target origami. This simplification, while not strictly true in all cases, is a reasonable first step for comparing anneal curves; it neglects the fact that the initial staple binding events are likely to be the longest (most stable) subsequences of staples that bind independently of the other subsequences. Small variations in the start/stop positions of these subsequences change the GC content and thus the $T_m$ of individual staples. We discuss these factors in detail in sections 1 and 4 of the supplementary data. This simplification allows for comparison between anneal curves such that over some temperature range, one can determine the curve with the higher fraction of formation (higher normalized fluorescence). Over that temperature range, the staples from that ‘dominant’ structure are more likely to be bound than those of the competing structure. One would also expect this dominant structure to be the most often observed by AFM of a competitively annealed sample. While this is a somewhat simplistic assumption, it is supported based on the powerful role of GC content in the hybridization of DNA. Areas of high GC content on the scaffold should anneal much earlier (i.e. display a higher $T_m$) regardless of cooperative effects, and staples which compete will share similar GC content.

For comparison with figure 5(A), as well as to see if the simplification described above can help in understanding chimera formation, several pure target origami anneal curves were compared (figure 5(B)). All four anneals in figure 5(B) were of 5 nM scaffold concentration. The top panel compares anneal curves for 2.5 × bundle staples (red line) and 5 × circle staples (blue line), both relative to the scaffold concentration, while the bottom compares 5 × bundle staples and 2.5 × circle staples. As such, they provide insight into 5 nM scaffold competitive anneals with 7.5 × total staple excess for a 66-33 and 33-66 bundle/circle competitive anneals. In the top panel of figure 5(B) the anneal curves cross at 59 °C; this indicates that for a 66-33 bundle/circle system the bundle curve is dominant (at a higher percent formation) for 78% of the folding process while for 32% of that range the circle curve is dominant. The bottom panel (curves cross at 61 °C) indicates that in the 33-66 bundle/circle system the circle anneal curve is dominant for 71% of the folding process while the bundle is dominant for 29% of the folding process. Overall, the top and bottom panels could be used to predict that, at 5 nM scaffold and 7.5 × total staple excess, a system with 33% bundle staples would fold using approximately 71% circle staples while a system with 66% bundle staples would form approximately 32% circle staples.

Figure 5(C) compares the anticipated behavior from fluorescence anneal curves above with the AFM data for 5 nM scaffold concentration in figure 5(A). There is promising agreement of these methods, however further work will be required to determine the conditions under which our simplification of competitive behavior can accurately predict AFM data from anneals. Within the scope of this study, it is reasonable to say that figure 5(C) indicates that intersection between independent, non-competitive anneal curves is necessary for chimera formation. If the anneal curves are clearly separate, with one structure consistently being more folded at any temperature, then chimera are unlikely to be observed. While we anticipate limits on the ability of anneal curves to predict chimera structural inheritance, the overlap of anneal curves appears to be a reasonable indicator of expected chimera formation.

From quantitative analysis of chimera AFM images, it is shown that chimera formation is highly dependent on parent staple ratio and annealing rate, with little dependence on scaffold concentration for the samples imaged. While exciting, such extensive AFM imaging data requires significant resource investment to gather. As such, it was compared to fluorescence anneal curves. The quantitative fraction of scaffold which folds into the circle was then compared to fluorescence data, indicating that at a minimum, one would not anticipate chimera to form when the anneal curves of the corresponding pure target structures did not intersect.
Anticipated chimera phase diagrams

While further work is necessary to determine how best to predict the conditions under which target structures will dominate or under which chimera will form, it is reasonable to use single target anneal data as guideposts to anticipate chimera formation. This approach is valuable as it leverages the high throughput available from equipment designed for real-time polymerase chain reaction (RT-PCR). With relatively simple analysis, this data was used to construct preliminary phase diagrams predicting structural outcomes from starting system conditions in competitive anneals. Compared to the high resource investment required to obtain similar information from AFM, this RT-PCR approach is likely to be a useful tool for initial evaluation of competitive annealing systems.

Fluorescence anneal data was gathered, (described in the methods) for the target bundle and circle origami separately of one another at 5 nM scaffold concentration. For each nanostructure seven staple excesses were used, relative to the scaffold concentration (2.5×, 5×, 7.5×, 10×, 15×, 20×, and 40×). Each system was gathered in triplicate and averaged after normalization. Figure 6(A) shows only five of the staple excesses form each structure for clarity, as one would expect, increased staple excess caused structure formation at higher temperatures. These curves were compared (as described in the Methods section) by checking for intersection as well as temperature ranges over which one structure predominates (as in figure 5(B)).

Systems were predicted to form bundle, circle, or to be chimeric. By comparing each staple excess anneal curve in one target structure to all of the anneal curves for the competing structure, 49 data points were gathered and plotted in the preliminary phase diagram shown in figure 6(B). The dotted lines were added as a guide to the eye and to indicate anticipated boundaries between plotted points. The horizontally striped region indicates conditions with less than 2.5× of either parent, in which polymerization/aggregation of origami is anticipated. The desirability of such diagrams is emphasized by the purple and blue lines in figure 6(B), which indicate the initial competitive anneals and the anneals on which quantitative image analysis was performed, a significant time investment. However, using RT-PCR equipment to obtain normalized anneal curves takes much less time and provides a useful indicator of chimera formation, particularly for new competitive systems.

As one would anticipate from previous samples and the anneal curves shown in figure 4, the diagram in figure 6(B) anticipates that the circle, in general, is more stable than the bundle as the circle takes up more space within the phase diagram. Additionally, it would suggest that as the total staple excesses increase relative to the

![Figure 6](image-url)

**Figure 6.** (A) Example normalized fluorescence anneal curves with varying staple excess. (B) Phase diagram predicted by anneal data. Red circles represent conditions under which the circle is expected to be dominant, blue squares represent conditions under which the bundle is expected to be dominant, black crosses represent conditions under which chimera and either cirlce or bundle origami are expected to form. (C) Example AFM images of origami from numerical points in (B).
scaffold, the system would be driven increasingly to form the circle nanostructure, although it is not clear whether the circle would ultimately displace either the chimera or bundle regions.

Figure 6(C) shows a small, but representative, selection of origami from the four numerical points in figure 6(B). As spot checks, rather than an exhaustive survey of the entire diagram, four x, y points on the diagram were selected for competitive anneals and AFM imaging. These points were at 60 x total staples (50-50 and 66-33 bundle/circle), 40 x total staples (75-25 bundle/circle), and 22.5 x total staples (45-55 bundle/circle). From these test points several observations can be made. Point 4 is reasonable for a pure bundle sample, as in all imaged samples containing any amount of circle staples, see the taxonomy in figure 2(A), the ends of bundle had some circle formation. This is likely due to a small overhang of the scaffold at either end of the bundle, designed to prevent base stacking. Point numbers 1 and 2 show that chimeric origami can still form at very high staple excesses, indicating that chimera formation is not linked to an insufficient excess of either parent staple pool. It is possible that the chimeric structures form as an equilibrium free energy minimum structure rather than as kinetically trapped state due to the frustrated folding pathway or that the kinetic trap remains even as strand displacement increases at high staple concentration. Overall these point tests indicate that the chimera phase region on the diagram is acceptable as a benchmark for chimera formation.

Regardless of the degree to which fluorescence anneal curves may be used to predict exactly how much of each parent is inherited, it has been shown that the anneal curves provide a convenient and quick method to anticipate conditions under which chimera might form for an arbitrary competitive annealing system.

Methods

Design, annealing and AFM

For this study two origami were designed in CADNANO, and analyzed using CANDO [28, 29]. Both used the miniM13 workbench scaffold [27]. The miniM13 scaffold is a 2.4 Kb workbench scaffold, as compared to the 7.4 Kb M13. The miniM13 allows rapid design and prototyping of new structures; this is valuable in studies where structures will be compared or design-property relationships characterized. The two origami used in this study were the circle and 6-helix bundle, also described in previous work [27]. The circle is a standard planar origami including skips to minimize curvature while the 6-helix bundle is a 3 x 2 helix bundle with a radically different routing pattern. Figure 2 shows the routing maps, the circle plots, and example AFM images for these nanostructures.

All samples were annealed in 50 mM sodium cacodylate buffer with 12.5 mM Mg acetate. All AFM images were gathered using an Asylum Cypher model AFM with biolever mini tips. Samples were imaged on mica in TAE Mg (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, 12.5 mM MgCl2) buffer supplemented with 5 mM NiCl2. First degree flattening was performed on all AFM images, which were then identically scaled and exported. ImageJ analysis of these images was performed using the particle counting tool; limits on particle size and circularity were set to ensure that neither streaks nor precipitates would be counted [30]. As the bundle structure is two helices tall, it is taller than the circle, allowing the two to be distinguished using thresholding tools in imageJ. By fitting ellipses each thresholded bundle in the particle counting tool, the length of the bundles could be easily quantified.

The images were then manually counted and both the number of fully formed origami and chimeric origami were counted. The number of chimeric origami counted was crosschecked back to the number found from thresholding. The major axes length of the thresholded bundle was used to weight the number of chimeric origami in manual count data. As each origami had a single scaffold molecule that was partially circle and partially bundle, this corrected the chimera data to be the fraction of scaffold folding into each design.

Fluorescence spectroscopy via RT-PCR equipment

Fluorescence melt/anneal data was gathered using the Quantstudio 6 system. Fluorescence samples were, unless otherwise specified, prepared in sets of 3 replicates each 20 ul of volume, of 5 nM scaffold concentration, with a 1 SYBR green molecule per 900 bases as per Sobczak et al [12], Rhodamine-X was added in 1:50 ratio by volume, and samples were buffered in pH 5.5 sodium cacodylate buffer. Sodium cacodylate buffer was chosen as its pH does not vary with temperature. The Quantstudio autodelta function with a cycle of 0.07 °C per step with a 20 s equilibration was used, leading to a rate of 0.21 °C min⁻¹, or 5.5 h for a single ramp between 25 °C and 95 °C. Each sample was annealed, melted and annealed again. Consecutive anneals were compared to ensure consistency. Using a python script replicate samples were averaged after normalized as per Sanford et al [31]. Fluorescence spectroscopy was gathered for 5 nM circle and bundle at 2.5 x, 5 x, 7.5 x, 10 x, 15 x, 20 x, and 40 x staples.
Anticipation of chimera formation from annealing curves

A rough estimation of chimera formation was created by comparing the anneal curves of the circle and bundle, then determining which was formed to a greater extent (higher fraction folded) at all temperatures across the anneal. As such, a prediction for 10× staples at 50% bundle (i.e. 5× bundle and 5× circle) would be made by comparing the anneal curve of 5× bundle staples with the curve from 5× circle staples. For the purposes of phase diagram prediction, the curves were classified as bundle dominant, circle dominant, or chimeric. The threshold for dominance was that the anneal curve for one structure should indicate formation at least 1–2 degrees higher than the other for at least 80% of the anneal curve. These points were then plotted as a phase diagram on the axes of total staple excess and fraction bundle staples.

Conclusions

In this study, we have shown that it is possible for competitive anneals between multiple scaffolded origami to result in discrete and orderly structures that are distinct from the parent origami. We have also performed preliminary analysis of these structures and provided several tools which may be of use in understanding other competitive systems. It has been shown that the circle-bundle system creates chimeras which favor the center of the bundle combined with the top and bottom of the circle. With this information and qualitative analysis of AFM data, tentative scaffold routing patterns for chimeric origami were created. These results provide further support for the importance of conformational entropy penalties in the scaffolded DNA origami folding processes. Through particle analysis in ImageJ, the average fraction of scaffold folding into the circle was quantified. From this analysis it was shown that, in this system, scaffold concentration has a minimal impact on chimera formation, while both the target staple ratio and the anneal rate had significant impacts on the chimeras formed. The importance of annealing rate indicates that strand displacement plays a major role in guiding the structures to an equilibrium shape, and that extremely slow anneals may be necessary to determine the true equilibrium chimera. Fluorescence annealing curves, obtained with Sybr green and RT-PCR equipment, were then used to provide a convenient and quick prediction of chimera formation.

Ultimately, these results provide an interesting way to examine the relationship between design of scaffolded DNA origami and folding, by examining the preferential formation of various substructures. Competitive anneals could be further leveraged to create new, more complex systems. The miniM13 workbench scaffold is an ideal testing ground for identifying design rules for these competitive systems; full M13 scaffold systems, as they are three times larger, are more likely to misfold and therefore may require nanostructure designs tailored specifically for competition. Such systems could provide very interesting network formation properties, as scaffold polymerization could be pre-programmed at locations between target structures and at chimera interfaces. Additionally, as the role of design is better understood, it opens the possibility to create systems which form different chimeras depending on whether they were kinetically trapped or annealed at equilibrium. Similarly, target structures could be designed with much longer ‘superstaple’ strands capable of altering the conformational entropy penalties in a strategic manner; the competition between such superstaples could influence the rest of the competition in interesting ways, opening the door to even more complex behavior. Overall, competitive anneals could provide an interesting way to increase the complexity and diversity of the DNA origami toolkit.

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