DNA Nanostructures

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Self-Assembly of DNA Origami and Single-Stranded Tile Structures at Room Temperature**

Zhao Zhang, Jie Song, Flemming Besenbacher, Mingdong Dong, and Kurt V. Gothelf**

During the past decade, the field of structural DNA nanotechnology has grown immensely,[1] especially since the invention of DNA origami,[2] and most recently the development of the single-stranded tile (SST) system.[3] Currently, DNA is used to create complex structures with precise addressability. These structures can serve as reliable templates for positioning of a variety of functional components,[4] and have also been used as devices to significantly narrow the gap between top-down and bottom-up approaches.[5] In a typical assembly experiment, the DNA strands are mixed in a magnesium-containing aqueous buffer, followed by thermal denaturation and a multi-step annealing procedure.[6] However, for many applications, it would be desirable to directly assemble DNA nanostructures at isothermal room temperature (RT), in particular for stepwise assembly pathways and for the integration of conjugates of other biological materials, such as proteins, that denature at elevated temperatures.

Formamide has been used to reduce the aggregation of long double-stranded DNA (dsDNA) at high temperature, for example, dsDNA scaffolds for origami.[7] In 2008, Jungmann et al. made use of the phenomenological equivalence between formamide concentration and temperature to achieve isothermal assembly of DNA origami by slowly lowering the concentration of formamide at room temperature.[8] Although the yield is comparable to the standard thermal-annealing methods, the setup of a dialysis system, which relies mainly on a microdialyzer, tends to be the experimental limitation. Herein, we have developed a simple, efficient and universal method for successfully assembling various complex DNA structures at RT, where a constant specific volume of formamide was part of the assembly buffer. In contrast to existing methods, no thermal cycler or dialyzer was required for the procedure, not even a preliminary thermal denaturation of the scaffold used for DNA origami. This general method was validated for the formation of both DNA origami and SST nanostructures, and more importantly, several unique advantages emerged and new assembly pathways were developed.

We first tested our room-temperature formamide incubation (RTFI) method to complete partially assembled DNA origami structures, by assembling part of the origami by the traditional annealing method, followed by addition of the remaining staple strands to the partially assembled DNA origami at RT in the presence of formamide. An equilateral triangular origami[2a] was chosen as the ideal two dimensional (2D) origami for this purpose, because it consists of three identical substructures (three edges) that are each scaffolded by a continuous single-stranded M13 DNA segment. Thus it was possible to study the assembly of each of the three substructures individually. Two pathways were tested, in which either one-third or two-thirds of the triangles were preformed by the traditional annealing method (Figure 1a, pathways 1,2) and the excess of staple strands were subsequently removed. RTFI assembly of the remaining parts of the triangular origami structures was performed at a series of final concentrations of formamide to optimize the procedure. Following incubation at RT overnight, the samples were imaged by atomic force microscopy (AFM) (Supporting Information, Figures S1,S2). For both pathways, 40% formamide (*v*/*v*) gave the highest efficiency of completion of the full triangle, with yields above 85% (Figure 1a inset and Table S1 for detailed statistics data).

The success of the RTFI method for shape completion encouraged us to apply the method to full structure assembly (Figure 1a, pathway 3). The yield of intact structures was highest when 40% formamide was used (Figure S3), providing a total yield of around 60% (Figure 1a inset). This yield is lower than Sobczak et al reported for origami assembly at constant temperature,[9] which may be due to the absence of the initial denaturation of the scaffold strand in the RTFI method. Low formamide concentration tends to result in incomplete folding and aggregation, while high formamide concentration only leads to fragmented structures. Further experiments revealed that overnight incubation was not required because four hours were sufficient to achieve a similar yield (Figure S4). The formamide was removed through buffer-exchange after assembly (Figure S5).

[*] Dr. Z. Zhang,[+] J. Song,[+] Prof. F. Besenbacher, Dr. M. Dong, Prof. K. V. Gothelf
Center for DNA Nanotechnology (CDNA) at the Interdisciplinary Nanoscience Center (iNANO), Aarhus University
8000 Aarhus C (Denmark)
E-mail: dong@inano.au.dk
kvg@chem.au.dk
J. Song[+]
School of Physical Science and Technology
Lanzhou University
Lanzhou 730000 (P. R. China)

[+] These authors contributed equally to this work.

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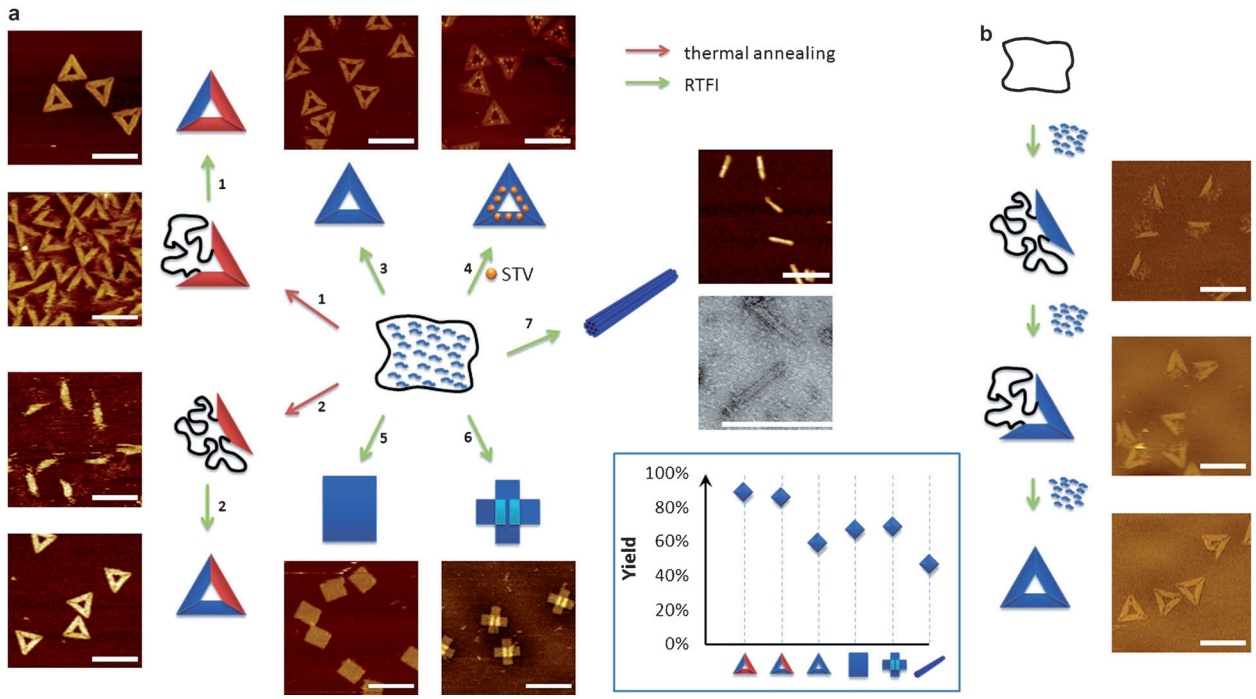

Figure 1. RTFI assembly of DNA origami. a) Scheme and AFM images of DNA origami assembled by the RTFI method through seven different pathways. Formamide (40%) was used for pathways 1–6, while 30% formamide was used for pathway 7. Inset: yields of assembly derived from AFM images (See Table S1 for detailed statistical data). b) Scheme and AFM images of stepwise assembly of DNA origami by RTFI. Subsets of staples were added into the reaction mixture sequentially, with the formamide concentration being kept at 40%. Scale bars = 200 nm.

A major advantage of RT assembly is that it may allow for in situ co-assembly of DNA origami with proteins, whereas most proteins would denature under the conventional annealing conditions. Hence, nine staple strands (three on each edge) were replaced with biotinylated staples and the mixture of staples was incubated with the M13 scaffold as well as streptavidin (STV) directly, in the presence of 40% formamide (Figure 1a, pathway 4). In this one-pot system, the incorporation yield of STV at the expected positions was 84%, which is consistent with another report where streptavidin was added after assembly of the origami structure.[10] Two other 2D structures, a rectangular origami[2a] and a cross-shaped origami[11] were also assembled under RTFI conditions in yields of up to 70% (Figure 1a, pathways 5,6).

Surprisingly, the RTFI method showed potential for mitigating the aggregation of DNA structures caused by base-stacking (Figure S6). Therefore, in addition to traditional strategies to prevent stacking of the origami structures, such as introducing 4T hairpin loops into the edge staples,[2a] the RTFI method provides another option but without changing the staple pool.

Generally, it is more challenging to assemble compact 3D origami structures than 2D structures,[2c] although annealing conditions were recently significantly improved by Sobczak et al.[9] To address the question of whether the RTFI method can be applied to 3D origami assembly, a 24-helix bundle (24HB) multi-layer origami packed on a honeycomb lattice[12] (Figure 1a, pathway 7) was chosen as a 3D prototype. Screening experiments showed that 30% formamide was the optimal concentration for the assembly of 24HB (Figure S7), and a longer incubation time (up to three days) was required. We attributed the lower optimal formamide concentration (30% versus 40%) for 3D origami structures to their different melting temperature profiles,[9] which stems from shorter seeding segments (14 bp versus 16 bp) and unit segments (7 bp versus 8 bp) in the staple patterns of a 3D design packed on a honeycomb lattice compared to the 2D design.

We further extended the RTFI method to the stepwise assembly of DNA origami. In previous studies, two-step assembly procedures were applied to create higher-order DNA structures,[2c,11,13] but the instability of DNA origami structures at high temperatures[14] prohibits repetition of the thermal cycles thus restricting the design. Three-step assembly was introduced for triangular DNA origami completion (Figure 1b) and was also tested for the rectangular origami (Figure S8). The final yield of stepwise assembly of DNA origami by RTFI ranged from 60% to 80%.

A highly modular strategy for self-assembly of DNA nanostructures was recently developed by Yin and co-workers. In this system, arbitrary 1D,[15] 2D,[3a] or 3D[3b] structures can be constructed from hundreds of distinct single-stranded tiles (SST) without the use of a scaffold strand. Herein, we have explored the assembly of SST structures under RTFI conditions. Formation of a complete 2D rectangular SST "canvas"[3a] and a right-triangle shape proved feasible after incubation with formamide (Figure 2, structures **1**,**2**). Optimization of the assembly conditions revealed 30% formamide as the optimal denaturant concentration, and the homogeneity and integrity of the structures were comparable

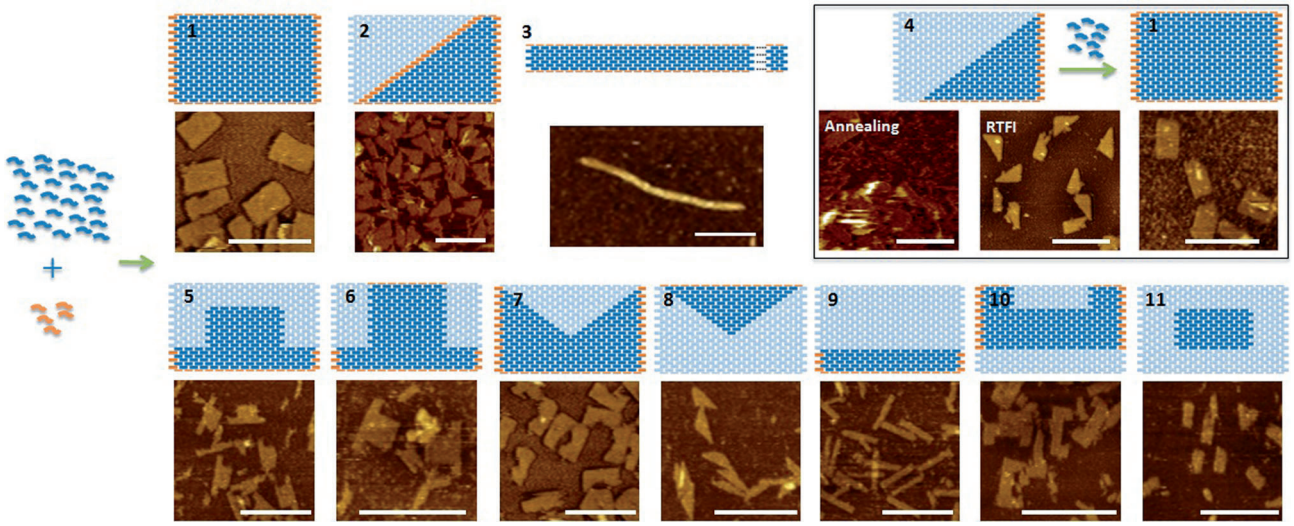

Figure 2. Scheme and AFM images of RTFI assembly of SSTs into 11 different shapes. Standard strands (32 nt, blue) and protector strands (16 nt, orange) for SSTs are shown. All the reactions used 30% formamide, except for the annealing reaction within the black box. In the black box, the left illustration is the SST right-triangle structure without protector strands in the diagonal (**4**). The right illustration is a full canvas (**1**) prepared from the RTFI-assembled structure **4** upon addition of the remaining SST tiles under RTFI conditions. Scale bars = 200 nm.

to those from standard annealing methods (Figure S9). An investigation of the effect of assembly time showed that one day was enough to bring the reaction close to equilibrium (Figure S10). The long SST ribbon structure **3**[15] also formed well under RTFI conditions, and its growth can be easily followed at RT (Figure S10).

The great advantage of the SST method is that arbitrary structures can be formed from the same canvas just by leaving out selected tiles, whereas a new set of staple strands is required for each new DNA origami structure.[3] However, in addition to the standard tiles in the SST canvas, a set of edge-protector strands are required for each structure to avoid aggregation (Figure S11), which not only increases the cost and inconvenience but also impairs the modularity of this method.

Because formamide is a denaturing agent, it also suppresses non-specific hydrogen bonding and hydrophobic effects, as observed by the decreased aggregation of the rectangular origami structure shown above. When the RTFI method was applied to the assembly of the right triangle from SST, without protector strands on the diagonal (structure **4**), the expected structures were indeed produced, whereas severe aggregation was observed when annealing was used (Figure 2 black box; Figure S12). Interestingly, when the remaining SST strands were added to the sample containing **4**, the full rectangle **1** was formed (Figure 2 black box). This indicates that the RTFI method also allows stepwise assembly of SST tile structures, which is an important step towards full modularity.

To further test the RTFI method for SST assembly, seven other shapes containing various degrees of edge protection were designed and assembled (Figure 2, structures **5**–**11**). According to AFM images, all the structures were formed without any noticeable aggregation, including structure **11** having no edge protection at all. However, the lack of edge protection makes the structures too labile to be purified by gel electrophoresis and therefore the yields could not be determined from gels.

Combining DNA origami and tile-assembly systems may be a strategy to scale up the size and complexity of DNA superstructures,[13e,16] as well as to nucleate algorithmic self-assembly.[17] Because the RTFI method works for assembly of both scaffolded and tile-based structures, as demonstrated above, we explored using the edge of a rectangular origami to incorporate a seed (adapter strands) to guide the growth of an SST structure, or an eight-helix ribbon (8HR) in particular (see Figure S13 for the design details).

Both a one-pot procedure and a stepwise procedure were adopted, and RTFI was used in the step of SST growth for both procedures (Figure 3a; Figure S14). AFM images showed that both the origami and the 8HR formed well, and they were joined as designed in most cases (Figure 3b; Figure S14). The two-step procedure resulted in a higher ratio of seeded ribbons than the one-pot procedure (Figure 3c), which is consistent with the hypothesis that a preformed seed will facilitate tile growth because of the presence of a nucleation barrier.[17c] Notably, the RTFI method also provided the opportunity to observe the process of nucleated tile growth at RT (Figure S15), without the need for thermal control on the AFM.[14,18]

The initial incentive to use formamide to assist assembly was inspired by two facts: formamide lowers DNA melting temperatures by functioning as a denaturing agent;[19] and formamide accelerates the rate of hybridization because it is an organic solvent.[20] However, when we substituted formamide with ethanol or DMF, which have been found to have similar properties,[20] no such facilitation effect could be observed (Figure S16). Based on the linear relationship between increasing formamide concentration and decreasing melting temperature (T_m),[21] we correlated the formamide condition to melting temperatures, where 40% formamide at RT corresponds to around 47 °C in a standard buffer (Fig-

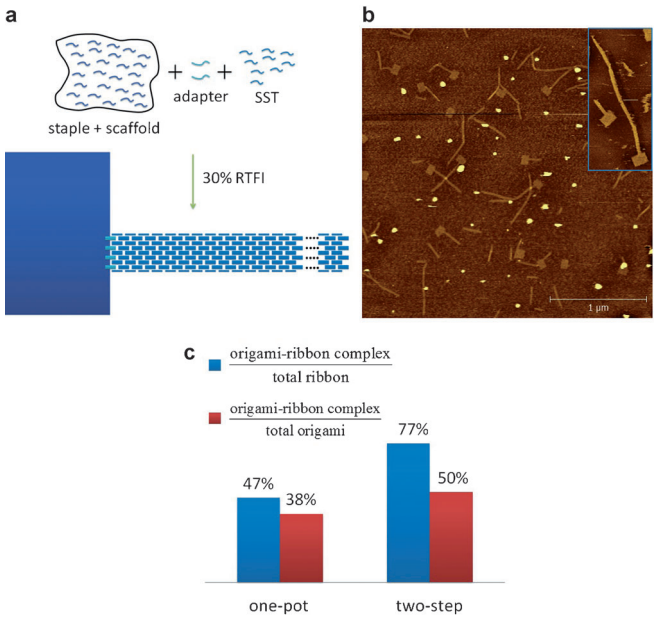

Figure 3. Two strategies for growing an SST ribbon on an origami edge. a) Scheme of a one-pot system, in which all the strands were mixed in the presence of 30% formamide directly. Scaffold (black), staples (purple), adaptors (cyan), SSTs (blue). b) AFM image of the assembly products in (a). Inset: magnified image. c) Percentage of the origami-ribbon complex to the total number of ribbons (blue) or origami (red). See Table S2 for detailed statistical data. The illustration and AFM image for a two-step procedure where annealing was used in the first step are given in Figure S14.

ure S17). To compare these conditions the assembly mixture was incubated at various constant temperatures between 45 °C and 60 °C overnight. The resulting yields were much lower than those from the RTFI method, although significantly higher than simple RT incubation in a standard buffer. Based on these results, we inferred that formamide plays a special role in the assembly process, probably owing to its capability to form hydrogen bonds with the bases, which competes efficiently with the H-bonds between Watson–Crick base pairs.[22] This unique competition mechanism reduces the activation energy barrier for both hybridization and denaturation reactions, therefore it drives the assembly forward even at constant temperature and disfavors mismatches at the same time. In support of this argument was the finding that a similar effect was obtained using urea (Figure S17), which is also a good H-bond donor and acceptor.

In summary, we have found that heating can be avoided in the formation of DNA origami and SST structures by using the RTFI method. Even the initial thermal denaturation of the M13mp18 scaffold for DNA origami scaffold was omitted. This new isothermal RT method has tremendous potential with a number of unique advantages such as 1) allowing stepwise assembly for expanding size and complexity, 2) assembly in the presence of a protein such as streptavidin, while maintaining its function, 3) reduction of blunt-end stacking, and 4) omitting protector strands in SST systems. Future applications may include in situ studies of the assembly pathways of DNA structures,[18] and reversible dynamic structure switching.[23] Furthermore, purified protein-DNA conjugates may be directly incorporated into origami during RTFI, which will make it more straightforward to prepare functional DNA nanostructures.

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