Sequential Pull-Down Purification of DNA Origami Superstructures

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Higher-order superstructures of individual DNA origami building blocks are frequently used in DNA nanotechnology in order to increase the structure dimensions and complexity. Here, a purification method is presented to specifically enrich a fully assembled superstructure out of an excess of substructures. The approach is based on pull-down reactions with magnetic beads, where superstructures are captured via an anchor strand on a specific terminus and then become separated from terminus-free structures. By carrying out several pull-down reactions sequentially on different termini, the full superstructures that possess all termini become finally enriched. The approach is demonstrated by purifying linear origami superstructures with up to nine monomers by two-sided pull-down reactions and a T-shaped superstructure in a three-sided pull-down reaction. In all cases, high recovery yields and purities are obtained. A crucial prerequisite for the sequential pull-down scheme is the establishment of highly specific, orthogonal sequence sets for capture, and anchor strands. It is expected that the introduced approach provides a useful and universal method to purify complex DNA origami superstructures with high specificity and yield and this way allows the massive parallel fabrication of nanostructures at high homogeneity.

1. Introduction

The field of DNA nanotechnology\cite{1,2} utilizes DNA as a building material based on the well-predictable Watson–Crick base-pairing interactions.\cite{3} In the past decade, rapid advances in DNA nanotechnology have enabled the fabrication of 2D and 3D nano- and sub-micrometer-sized structures with large diversity and complexity at nanometer resolution.\cite{4,5} Among several pioneering techniques,\cite{6-10} the scaffold-based DNA origami technique\cite{10,11} has particularly achieved to assemble defined high-molecular-weight structures at large yields. Site-specific anchors on the DNA nanostructure surface allowed furthermore to bind and arrange a multitude of other nanomaterials, thereby promoting new applications in biological research,\cite{12,13} nanoelectronics,\cite{14,15} plasmonics,\cite{16,17} and therapeutics.\cite{18-20}

The high yield at which DNA origami structures can be assembled is based on the integrity that the \(\approx 8000\) nt long single-stranded scaffold DNA naturally provides. However, a predefined scaffold length sets an upper limit for the molecular weight of such structures. The usage of larger single-stranded scaffold DNA that can be produced\cite{21,22} is associated with significantly increased costs and design efforts. An alternative, simpler and more frequently used approach to fabricate large-scale DNA origami structures is therefore the hierarchic assembly of DNA origami monomers.\cite{23} Assembly of DNA origami superstructures is either based on interactions between complementary DNA strands\cite{24,25} or on the stacking interactions between DNA ends,\cite{7,26} whereby the latter method provides higher efficiencies. For stacking interactions either blunt DNA ends or “sticky” ends, i.e., short single-stranded overhangs, that invade the scaffold of the partner structure, are used.\cite{27,28} To obtain DNA origami superstructures of a well-defined size, one can use a sufficient number of unique interfaces between origami monomers.\cite{29,30} Alternatively, one can assemble superstructures with a discrete rotational symmetry using a periodic interface arrangement which results in a self-limiting hierarchical oligomerization upon closure of the full turn of the structure.\cite{28} Such a bottom-up formation of superstructures has, however, limited yields\cite{29} such that preparations often contain a significant amount of substructures.

For many applications, it is necessary to achieve a high purity of the final superstructures. A way to overcome the limited assembly efficiencies would be provided by a universal purification method with a high recovery yield. So far, several purification methods have been established to purify DNA origami structures from the excess of the much smaller staple oligonucleotides,\cite{31} such as polyethylene glycol (PEG) precipitation,\cite{32} agarose gel electrophoresis,\cite{33} gradient ultracentrifugation,\cite{34} ultracentrifugal filters,\cite{35} and size exclusion columns.\cite{35} Among these methods, only agarose gel electrophoresis is able to separate DNA origami...
superstructures from incomplete substructures. However, due to the required thermal, chemical, and/or mechanical destruction of the agarose gels, this method has a low recovery yield, is prone to damage the DNA structures, and retains contaminants such as residual agarose or DNA staining dyes.

As an alternative purification method for DNA nanostructures, affinity-based pull-down of sample molecules from solution using magnetic beads has been recently established.\cite{36} This method is noninvasive and provides high recovery yields of up to 70%.\cite{37} Anchor strands on the DNA origami structures support the binding to magnetic beads that are functionalized with complementary capture strands. Bead pelleting in a magnetic field gradient allows the exchange of the solution and thus the removal of the excess of unbound molecules, which can be further improved by additional washing steps. Toehold-mediated strand displacement\cite{38} is finally used to displace the anchor from the capture strands and to release the origami structures from the beads. The pull-down method allows purifying DNA origami nanostructures not only from excess staple strands but also from nanoparticles, proteins, or other functional nanomaterials.\cite{37} So far, this method, however, does not support the purification of DNA origami superstructures from incomplete substructures.

Here, we expand the potential of the pull-down method to allow for the first time a high-yield purification of DNA origami superstructures. The crucial component of our method is the application of several sequential pull-down steps at opposing termini of the superstructures. It is based on the idea that only complete superstructures possess all termini. To allow the specific pull-down of a desired end in a given step, we use different anchor-capture sequences that are orthogonal to each other. We apply our method to linear origami superstructures (Figure 1) that are first pull-down purified using corresponding anchors at one end and then at the other end. This allows a successful purification of pentamer, octamer, and nonamer structures. Beyond this, we demonstrate the purification of junction superstructures involving pull-downs from three different ends. We carefully fine-tuned the purification protocol to achieve high specificity and high yields. Overall, our method should be applicable to many DNA origami superstructures in a highly versatile manner.

### 2. Results and Discussion

As a model sample, we used a previously reported DNA origami tube structure\cite{14} that can be assembled into a linear pentamer using four specific interfaces (Figure 2a). A tube monomer consists of 64 DNA helices of ≈40 nm length that are arranged on a square lattice to form a square-shaped cavity of $6 \times 6$ helices (Figure S1a, Supporting Information). The tube monomer possesses a polarity resulting in different left and right ends. The specific interactions between a left and a corresponding right end were achieved by a combination of repulsive helix ends with dangling single stranded DNA (ssDNA) ends and attractive helix ends with short ssDNA overhangs that can invade the scaffold at the other interface side. The combination of different overhang types and patterns to locate attractive and repulsive ends allowed to define four orthogonal interfaces A, B, C, and D (Figure S1a, Supporting Information) that supported the self-assembly of a specific pentamer at 76% yield.\cite{30}

To support pull-down purification, the left- and the right-most monomers carried specific anchor strands as staple extensions on their left and right ends, respectively (Figure 1). The terminal 20 nt of the anchors were complementary to a 20 nt stretch on corresponding capture strands. The capture strands contained in addition a 15 nt toehold sequence for the loading of a corresponding release strand as well as a biotin modification for immobilization on streptavidin-coated magnetic beads (see Table S1, Supporting Information, for strand sequences).

Before starting a pull-down reaction, magnetic beads were first densely coated with capture strands. We found that the used magnetic beads supported a maximum loading capacity of 100 pmol capture oligonucleotides per 10 µL magnetic beads (10 mg mL$^{-1}$, see Table S2, Supporting Information) that we then applied for the following experiments.

We next carried out a two-sided sequential pull-down reaction on the pentamer structure with anchor strands 1 and 2 on
Using the stable pentamer, we simulated a more complex purification reaction that contained large fractions of monomeric and trimeric substructures. This was achieved by mixing the five monomers in unequal stoichiometric amounts (Figure S2, Supporting Information). The substructures were equally well captured and released as the full structure during the right-end capture and release steps C1 and R1. This indicated a strong unspecific binding of the DNA tubes to the capture strands. The left-end pull-down reaction exhibited a slightly higher specificity during capture C2 but retained a large fraction of unspecific substructures (Figure S2, Supporting Information).

We identified dangling ssDNA ends of the attractive and repulsive helix ends as a potential cause for the unspecific binding of the substructures. We tested three different sequence sets of capture-anchor strands for their nonspecific binding of tube monomers (Figure S3a and Table S1, Supporting Information, for strand sequences) including sequence set 1 for the right end and two sets for the left end (sequence sets 2 and 3). Beads coated with capture strand 3 (Figure S3b, Supporting Information) were highly specific for binding tubes with anchor strand 3 rather than anchor strands 1 and 2, while beads with capture strand 1 (Figure S5b, Supporting Information) and 2 (Figure S3b, Supporting Information) could also bind tubes with non-complementary anchor strands. To increase the specificity of the capture strands, we blocked unused capture strands on the beads during the capture step with competitor oligonucleotides that were complementary to the capture region (Figure S4a, Supporting Information). At high amounts of competitors, both specific and nonspecific capture was blocked. When lowering the amount of competitor, an optimum between binding tubes with complementary anchors and rejecting tubes with non-complementary anchors could be found. The optimal specificity was achieved for ratios between competitor and capture strands of 1:1.5 and 1:8 for sequence sets 1 and 2, respectively (Figures S4 and S5, Supporting Information). Notably, sequence set 3 could still sufficiently bind tubes with specific anchors for a competitor-to-capture strand ratio of 1:8 (Figures S3 and S4, Supporting Information). The recovery yield of captured and released tube monomer was 80% for anchor 1, 32% for the anchor 2, and 70% for anchor 3. We furthermore optimized the buffer composition and the incubation times for both the capture and release steps. Optimized conditions are given in the Experimental Section. To understand the differences in the required competitor amounts for optimal specificity between the different sequence sets, we determined melting temperatures and the GC contents of the capture-anchor dimers (using Integrated DNA Technologies (IDT) server). Furthermore, we calculated the free energies of heterodimers between non-complementary capture and anchor strands as well as between capture strands and repulsive overlaps at the superstructure ends. We did not find significant differences between the different sequence sets, such that we attribute the different specificities to complex multivalent interactions between capture strands on the beads and the repulsive overlaps at helix ends.
After improving the stability of the superstructures, the capture strand specificity, and the reaction conditions, we repeated the two-sided pull-down reaction of the pentamer sample with anchors 1, 2, and a generic 1:4 ratio of competitor over capture strands (Figure 2a). Before the purification, the sample contained the full pentamer as well as monomer and trimer substructures. After the first capture step C1 of the right end, agarose gel electrophoresis of the supernatant revealed a specific decrease of the intensity of the pentamer band, i.e., specific capture of the pentamer (Figure 2b). The first release step of the bound structures from the magnetic beads was initiated by adding the release strand of the sequence set 1. This provided an enrichment of the pentamer structure over the substructures (Figure 2b) shown by quantitative analysis of the band intensities in agarose gel electrophoresis of the different structures (Figure 2c). The released sample was then subjected to capture step C2 of the left end using an optimized 1:4 ratio of competitor over capture strands. As in the previous capture step, the amount of pentamer structures was reduced within the supernatant (compare R1 and C2 sample in Figure 2b,c). Incomplete pentamer capturing is most likely caused by inhibition of anchor binding due to the added competitor. After inducing release step R2 using release strand 2, the sample contained tube pentamer structures without any noticeable contributions from monomer and trimers (Figure 2b,c). This demonstrates a successful purification of the desired pentamer structure from an excess of substructures using our two-sided pull-down protocol.

We determined a total recovery yield of 40% ± 2% (intensity of purified pentamer band vs unpurified pentamer band) and a purity of nearly 100% (intensity of purified pentamer band vs total intensity) for the pentamer purification (see Figure 2b). We furthermore analyzed the sample before and after purification using transmission electron microscopy (TEM). The TEM images confirmed that monomer and trimer substructures were abundantly present before purification (Figure 2d) while after purification, the pentamer became highly enriched (Figure 2e, overview image Figure S6, Supporting Information). The purity of the pentamer structures from the TEM images was 93% ± 5% (N = 604, comparing tube monomers in pentamers vs total tube monomers). The excess of release strands in the purified sample (see bright bands at the bottom of Figure 2b) was seen in the TEM images as disordered aggregates (see red arrows in Figure 2e). The release strand aggregates could be removed by PEG purification.[32] TEM imaging confirmed the successful removal of the excess release strands (Figure S7, Supporting Information) albeit the percentage of the pentamer structure appeared to be reduced to 74% ± 3% (N = 223) indicating that the pull-down purification is less harsh to origami superstructures compared to PEG precipitation.

To further test our purification approach, we purified larger linear octamer and nonamer superstructures with molecular weights of 43 and 48 MDa. Structure assembly was based on the same four interfaces (A, B, C, and D) with 5 nt sticky ends as used for pentamer formation. For octamer formation, four tube dimers were preformed using interface A followed by dimer linkage to an octamer using interfaces B, C, and D (at a 1:2:2:1 ratio of dimers, see Figure 3a).[30] Similarly, nonamers were formed from three preformed trimers using interfaces C and D (at a 1:2:1 ratio of trimers) in which the individual trimers were...
formed using interfaces A and B.Both structures contained anchors 1 and 2 at their right- and left-most monomer, respectively (Figure 3a). Dimer and tetramer substructures were observed for the octamer sample while trimer and hexamer substructures were seen for the nonamer sample (Figure 3b). The two-sided sequential pull-down purification was first applied to the right side and then to the left side of the structures. After completing the two capture steps C1 and C2, the full superstructures appeared always to be depleted in the supernatant (compare Figure 3b; Figure S8a, Supporting Information) indicating specific end capture. After the first release R1, the full structures became slightly enriched, but they were strongly enriched after the final release R2. The predominant components were the desired full octamer and nonamer structures (Figure 3b). Using quantitative analysis of the agarose gels, we determined the purity to be 81% ± 2% and 65% ± 2% and the recovery yield to be 37% ± 2% and 62% ± 2% for the octamer and nonamer structures, respectively. Additional TEM imaging verified the presence of different tube lengths in the mixture before purification (Figure 3c,e; Figures S9 and S10, Supporting Information). After the sequential pull-down purification, the octamer and nonamer structures became highly enriched in the TEM images (Figure 3d,f; Figures S9 and S10, Supporting Information). The purity was 86% ± 2% (N = 150) for octamer and 79% ± 3% (N = 139) for nonamer structures as counted from the TEM images in agreement with the gel-based analysis.

Conceptually, the sequential pull-down reaction can be extended to a higher number of purification steps. To test this, we used a T-shaped superstructure containing three different ends that was assembled from three tube monomers and one junction element (Figure 4a). Once again, attractive helices with 5 nt sticky ends were utilized for the interfaces. Each terminus carried a single specific anchor (anchors 1, 2, and 3, see Figure 4a). The unpurified sample (assembled from 2:2:2:1 mixture of three tube monomers and the junction element) contained as dominant species tube monomers in addition to dimeric and trimeric substructures and the full tetrameric junction (lane U in Figure 4b). For purification, three subsequent capture-release steps were applied using magnetic beads coated with either capture strands 1, 2, or 3. Already after the first release R1, the full T structure became dominant (Figure S8b, Supporting Information). The purity was further increased after the second release R2 (Figure S8b, Supporting Information) and the final release R3 (lane P in Figure 4b). This demonstrated a successful three-sided sequential pull-down reaction. The purity of the full T structure was 69% ± 2% and the recovery yield was 66% ± 2% as analyzed from the band intensity in agarose gel. TEM imaging confirmed again the presence of a high percentage of substructures before purification (Figure 4c) and the dominant presence of the full T structure after the three-sided pull-down reaction (Figure 4d; Figure S11, Supporting Information). The final purity determined from TEM images of the T structure was 86% ± 3% (N = 104).

Figure 4. Three-sided sequential pull-down of a T-shaped superstructure. a) Scheme of the T-shape structure with specific anchor strands at each of the three ends. b) Analysis of the three-sided pull-down of the T-shaped structure using gel electrophoresis. Lanes L denote the 1 kb plus size marker and S the single-stranded p8064 scaffold DNA. U indicates the unpurified sample containing a minor fraction of full-length and a large fraction of smaller multimers. P indicates the purified sample after the three-sided sequential pull-down. c) TEM image of the unpurified sample. d) TEM image of the purified sample after the three-sided sequential pull-down. Scale bar: 100 nm.

3. Conclusion

In summary, we introduced a universal method to specifically purify origami superstructures with high recovery yield. The method is based on sequential pull-down steps using magnetic beads as solid support to remove incomplete assembly structures. The pull-down steps were applied to the different termini of the structures. Complete structures possessing all termini could be highly and specifically enriched out of an excess of incomplete substructures. We successfully carried out two-sided sequential pull-down purifications on linear pentamer, octamer, and nonamer structures reaching purities between 65% and 100% as determined by agarose gel electrophoresis (between 79% and 93% as determined with TEM). Achieved recovery yields were between 37% and 62%. We furthermore carried out a three-sided pull-down reaction of a T-shaped superstructure reaching a purity of 69% as determined from gel electrophoresis (86% as determined from TEM) and a recovery yield of 66%. The purities and recovery yields of all structures that were purified in the work are summarized in Table S3, Supporting Information. Despite employing a multiple pull-down reactions, the obtained recovery yields were similar as obtained for single-step pull-down reactions.17 Compared to unpurified superstructures that were assembled at optimal monomer ratios, the purity increased significantly after the sequential pull-down reaction. For example, the purities of assembled octamer and nonamer samples of 46% and 41% increased to 86% and 79%, respectively. We note that our method is generally extendable to an even larger number of sequential pull-down steps. The crucial point for successful multistep purification was a high specificity of the capture-strand-coated magnetic beads towards the addressed origami.
monomer. Unspecific monomer binding that alleviated the specificity was suppressed by adding an optimized amount of competitor strands. Since the three different anchor-capture strand sets displayed relative differences in their specificity, it is conceivable that a further selection of optimized sequence sets, e.g., from a large pool of sequences, can still improve specificity and yield of the pull-down reactions. This would also facilitate sequential pull-downs with an even larger number of steps.

Overall, we expect that this method is highly useful for the purification of a large variety of DNA origami structures and other DNA nanostructures. We note that the purification also separates the desired superstructures from other nanomaterials, such as inorganic nanoparticles, which further broadens the applicability of the introduced method.

4. Experimental Section

DNA Origami Superstructure Assembly: DNA origami tubes with corresponding interfaces on the L- and the R-end were designed with the software-tool CaDNAno[39] using a square lattice[40] for the DNA helix arrangement. The origami structures were assembled in folding buffer (FB) containing $5 \times 10^{-3} \text{ M Tris–HCl}, 1 \times 10^{-3} \text{ M EDTA}$, and $11 \times 10^{-3} \text{ M MgCl}_2$ (pH 8.0) following a one-pot assembly protocol.[41] Subsequently, the tube monomers were purified using precipitation with PEG to remove excess staples.[42] For superstructure assembly, the different origami monomers carrying specific end staples that define the desired interfaces were mixed at the indicated stoichiometries in FB supplemented with $350 \times 10^{-3} \text{ M NaCl}$ and were left for incubation overnight. Assembly of octamer and nonamer structures occurred in two separate assembly steps. First, four different dimers involving the interface A were assembled for octamer fabrication while three different trimers involving the A and B interface were assembled for nonamer fabrication (Figure 3a). Subsequently, the full-length structures were fabricated by mixing and incubating the preassembled dimers (at a 1:2:2:1 ratio) or the trimers (at a 1:2:1 ratio). The prepared tube superstructures were analyzed by gel electrophoresis in 0.8% agarose gels and 0.5× TBE buffer supplemented with $11 \times 10^{-3} \text{ M MgCl}_2$ at 3.5 V cm$^{-1}$ for 180 min. Gels were stained in a 0.0002% ethidium bromide solution and then imaged using a ChemiDoc MP imaging system. For TEM imaging, 5 µL of a $2 \times 10^{-9}$–$5 \times 10^{-9}$ M solution of origami structures was placed onto glow-discharged carbon-coated TEM grids for 5 min. The sample was subsequently stained using a filtered 2% solution of uranyl formate. The droplet of uranyl formate was immediately removed, followed by a second addition of uranyl formate for 10 s. TEM imaging was performed on a Jeol JEM2100Plus transmission electron microscope at 200 kV.

Capture Procedure: For a typical experiment, 20–50 µL streptavidin-coated magnetic beads (Thermo Fisher Scientific) were washed three times with 500 µL washing buffer (WB, $10 \times 10^{-3} \text{ M Tris–HCl}, 1 \times 10^{-3} \text{ M EDTA}$, $12 \times 10^{-3} \text{ M MgCl}_2, 5 \times 10^{-3} \text{ M NaCl},$ and 0.05% Tween 20). This involved bead pelleting within a magnet rack, supernatant removal, and resuspension of the washed magnetic beads at 10 times the original bead stock volume. The mixture was incubated for 1 h at room temperature while kept under constant rotation at 25 rpm. The coated beads were washed against three times with 500 µL WB to remove the unbound capture oligonucleotides.

In order to test the capture specificity of origami monomers, 20 µL of the magnetic bead stock was applied, followed by the abovementioned capture strand decoration procedure. The capture strand-coated magnetic beads were pelleted and resuspended in 10 µL WB supplemented with $10 \times 10^{-8}$–$10 \times 10^{-6}$ M origami monomers. For improving the specificity, WB was replaced with a 10 µL solution of competitor strands whose amount corresponded to the indicated stoichiometric ratios over initially the applied capture strands.

For the purification of tube superstructures, 50 µL bead stock solution was washed and resuspended in 25 µL WB supplemented with 25 µL of a solution of superstructures with an origami monomer concentration of $20 \times 10^{-9}$ M. Optimized competitor ratios for the different superstructures were also applied. A ratio of 1:4 between competitor and capture strands was applied for the pentamer structure, while a ratio of 1:10 was used for the other superstructures. The mixture of capture beads and origami nanostructures was incubated overnight at room temperature at constant rotation at 25 rpm. Beads were then pelleted and the supernatant was analyzed by agarose gel electrophoresis. The beads were then washed three times with 500 µL washing buffer.

Release Procedure: For the release of the origami nanostructures from the capture beads, 20 $\times 10^{-6}$ M release oligonucleotides (in $10 \times 10^{-3} \text{ M Tris–HCl}, 1 \times 10^{-3} \text{ M EDTA}, 6 \times 10^{-3} \text{ M MgCl}_2,$ and $300 \times 10^{-3} \text{ M NaCl}$) was added to the washed and pelleted magnetic beads at the same volume as the applied bead stock, which corresponds to a twofold molar excess over the initially applied capture oligonucleotides. The mixture was incubated overnight at room temperature while constantly rotating at 25 rpm. Beads were pelleted and the supernatant with the released nanostructures was used for further analysis and/or subsequent pull-down reactions from different termini that repeated the same protocol.

Analysis of Sample Purity and Recovery Yield: To determine the sample purity after the final release from agarose gel images, the band intensity for the full superstructure was normalized by the total intensity of all bands in the product lane (P). To determine the recovery yield, the band intensity for the full superstructure after purification (lane P) was normalized by the band intensity for the full superstructure before purification (lane U). Band intensity from agarose gel images was quantified using the Image Lab software of the ChemiDoc MP imaging system and ImageJ. Prior quantification, a 10 mm rolling disk filter was applied to the images for background subtraction. After each release step of a sequential pull-down purification, 10 µL of the 50 µL release volume was used for gel analysis. The final recovery yield was corrected to account for the product loss.

The sample purity was also determined from the TEM overview images by normalizing the number of origami monomers found in full superstructures by the total number of monomers in the images.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.Y. and J.T. contributed equally to this work. This work was supported by the Deutsche Forschungsgemeinschaft within the Cluster of Excellence Center for Advancing Electronics Dresden (cfaed/TU Dresden) as well as grant SE 1646/8-1 to R.S. The authors acknowledge Björn Högborg, Fatih Nadi Gör, and Thorsten-Lars Schmidt for useful discussions. The authors thank David Poppitz for the training and support of the TEM imaging.

Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.
Keywords
DNA nanotechnology, hierarchical self-assembly, magnetic beads, purification, solid support

Received: November 16, 2020
Revised: January 19, 2021
Published online: March 16, 2021

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