

Nanofabrication Yields. Hybridization and Click-Fixation of Polycyclic DNA Nanoassemblies

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DNA has become a reliable material for nanoscale construction, a technological application well outside the realms of biology and its natural use as information storage of the genetic code. In his pioneering work, Seeman has demonstrated that the unique structural features of DNA may be utilized to fabricate complex constructs by sheer self-assembly on the nanometer scale, proving the potential of applying a molecular perspective to nanoscience with a bottom-up approach. With the development of different robust crossover motifs, oligonucleotides may be assembled into remarkable crystal-like structures, demonstrating that it is possible to create large-scale nanostructures with small DNA building blocks.^{1–4} Since then, various strategies in DNA nanotechnology have emerged and a large variety of geometric shapes have been created using DNA molecules, in 3D as well as in 2D.^{5–11} One strategy that has become successful for nanoscale fabrication is “DNA origami”.^{12–22} This approach involves the controlled folding of a long viral DNA molecule into a desired structure. The folding process is regulated by numerous short oligonucleotides, complementary to specific parts of the genomic sequence. Adopting this strategy, it has been possible to fold the several kilobase long single-stranded DNA molecule of M13 bacteriophage into various shapes. After initial folding into patterns in 2D,^{13–15} researchers have recently been able to create 3D shapes using the origami strategy.^{12,16–21} The field of DNA nanotechnology has seen not only the advance of architecture strategies for static nanoscale construction but also the development of nanomechanical devices involving dynamic processes, exploring the possibility of creating DNA machines.^{23–31} There are several examples where structural shifts of a DNA construct have been utilized

ABSTRACT We demonstrate the stepwise assembly of a fully addressable polycyclic DNA hexagon nanonetwork for the preparation of a four-ring system, one of the biggest networks yet constructed from tripodal building blocks. We find that the yield exhibits a distinct upper level <100%, a fundamental problem of thermodynamic DNA assembly that appears to have been overlooked in the DNA nanotechnology literature. A simplistic model based on a single step-yield parameter y can quantitatively describe the total yield of DNA assemblies in one-pot reactions as $Y = y_{\text{duplex}}^n$, with n the number of hybridization steps. Experimental errors introducing deviations from perfect stoichiometry and the thermodynamics of hybridization equilibria contribute to decreasing the value of y_{duplex} (on average $y = 0.96$ for our 10 base pair hybridization). For the four-ring system ($n = 31$), the total yield is thus less than 30%, which is clearly unsatisfactory if bigger nanoconstructs of this class are to be designed. Therefore, we introduced site-specific click chemistry for making and purifying robust building blocks for future modular constructs of larger assemblies. Although the present yield of this robust module was only about 10%, it demonstrates a first step toward a general fabrication approach. Interestingly, we find that the click yields follow quantitatively a binomial distribution, the predictability of which indicates the usefulness of preparing pools of pure and robust building blocks in this way. The binomial behavior indicates that there is no interference between the six simultaneous click reactions but that step-yield limiting factors such as topological constraints and Cu(I) catalyst concentration are local and independent.

KEYWORDS: DNA nanostructure · click chemistry · supramolecular assembly · yield analysis · fixation technology

to repetitively switch a device between geometrically different states, a process driven by, for example, an ionic gradient²⁵ or the free energy of hybridization, using oligonucleotides added as “fuel”.^{23,26} The repetitive motion is based on back-extraction of the fuel oligonucleotides by addition of complementary oligonucleotides forming more stable duplexes.^{27,28} In this way, contractile force walking devices have been constructed, moving unidirectionally along a track.^{29–31}

Evidently, there is great potential in using DNA as a material for programmable nanoscale fabrication. Using crystal-like arrays of crossover motifs, it is possible to create quite large structures from a bottom-up perspective. However, one feature of biological DNA is lost in these artificial repetitive

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structures, *viz.* the inherently information-rich high complexity of the polymer, a feature that could be important in certain applications. One can envision future applications requiring a spatial precision that is not achievable with repetitive crystal-like assemblies, and in which each position in the assembly is unique and may be addressed individually. The concept of an addressable grid has been demonstrated before in various contexts, such as with the construction of a DNA-tile-based “molecular pegboard”^{32,33} or more recently using the DNA origami approach.³⁴ With an alternative fabrication path, working with smaller sub-units, it would in principle be possible to create structures with similarly distinct features but with higher variability and resolution on a truly molecular scale, compared with the origami strategy which is restricted by the several kilobases large genomic template sequence. Our strategy is to assemble a fully addressable DNA network in two dimensions with the smallest practical unit of DNA, that is, one turn of the helix, as a basis for the assembly. By using a small synthetic node with D_{3h} symmetry (Figure 1, bottom) and orthogonal protection group chemistry, we have previously reported the assembly of a cyclic hexamer, “DNA-benzene”,³⁵ and a bicyclic construct, “DNA-naphthalene”,³⁶ where each edge has a unique sequence and, thus, can be specifically addressed. The addressability may allow specific functionalization patterns with precise positioning of nanoparticles^{37–40} or chemical reaction centers.^{34,41}

Here we present the formation of an unsymmetrical four-ring network assembled in a one-step annealing process (Figure 1, top). As in our previous reports, each side in the hexagonal network constitutes only 10 nucleotides, corresponding to a side length of 3.4 nm assuming B-DNA helical structure. The C_4 linker, connecting the oligonucleotides to the benzene node, should create sufficient flexibility to avoid possible distortions due to the 10.5 bp helical pitch. Each side in the network has a unique sequence, giving specific addresses over the entire structure. This is achieved by designing 10 base sequences that are complementary to only one other sequence in the system and orthogonal to all others. The orthogonality condition is here defined as a mismatch criterion of ≥ 4 (for any strand alignment) between two noncomplementary 10 base sequences. There are two sides in the present construct that are not unique. One three-way branched oligonucleotide is deliberately used twice in the assembly, the reason being that we want to investigate assembly robustness and the possibility to incorporate generic nodes in the network and still maintain structural integrity. Finally, we considered the yields obtained for the primary hybridization steps and of the total assembled structure, as well as that of the product from a click-fixation technology,⁴² from a theoretical point of view with the objective of paving the way for

alternative approaches to conventional one-step assembly of DNA nanostructures. We find that the yields of these types of reactions may be described in simple statistical terms. Models explaining the fundamental behavior of nanosystems based on DNA self-assembly have so far been absent in the literature. Thus, answering questions concerning the thermodynamic aspects of DNA nanotechnology is of vital importance for efficient and reliable fabrication of intelligent nanomaterials.

RESULTS AND DISCUSSION

We have previously demonstrated the assembly of two hexagonal cells fused together, denoted DNA-naphthalene.³⁶ This serves as a reference structure in the current study and the starting point for stepwise assembly of the new structures to be proven. By starting with a known structure and adding stepwise one building block at a time, it is possible to follow the assembly of larger constructs by the sequential retardation of bands in gel electrophoresis. In this study, we construct a network of four hexagonal cells fused together, assembled by 17 nodes and 31 duplexes formed in a one-step self-assembly.

Before formation of the four-cell network, we demonstrate the stepwise assembly of the two possible three-cell constructs, denoted “DNA-anthracene” and “DNA-phenalene”. The stepwise assembly of DNA-anthracene is shown in Figure 2. The starting point is the two-cell structure DNA-naphthalene (A), seen in the left lane on the gel. The following lanes contain a sequential addition of new three-way oligonucleotides (B–F), following the schematic to the left of the gel, ending with the DNA-anthracene (G). Each addition of an oligonucleotide causes a small but distinguishable retardation of the band on the gel, due to the change in geometry giving a more rapidly increasing hydrodynamic friction coefficient compared to increased DNA charge force when building larger constructs. On the basis of the stepwise decrease in mobility of each sequential construct, we can conclude that the DNA-anthracene has successfully been assembled. The intermediate structure F exhibits three separate bands corresponding to binding of one, two, and three nodes to the two-cell construct (a blow-up of the bands with increased resolution is shown in Supporting Information, together with an intensity profile analysis). A similar pattern can be seen for samples D and E on the gel. Both display two adjacent bands corresponding to one node being bound or not. This poor hybridization yield of intermediate structures has been seen in this kind of system before and is believed to originate from the single-stranded sequences of the three-way oligonucleotides (see below). By contrast, the totally ring-closed product of DNA-anthracene (G) displays one distinct band with no other large

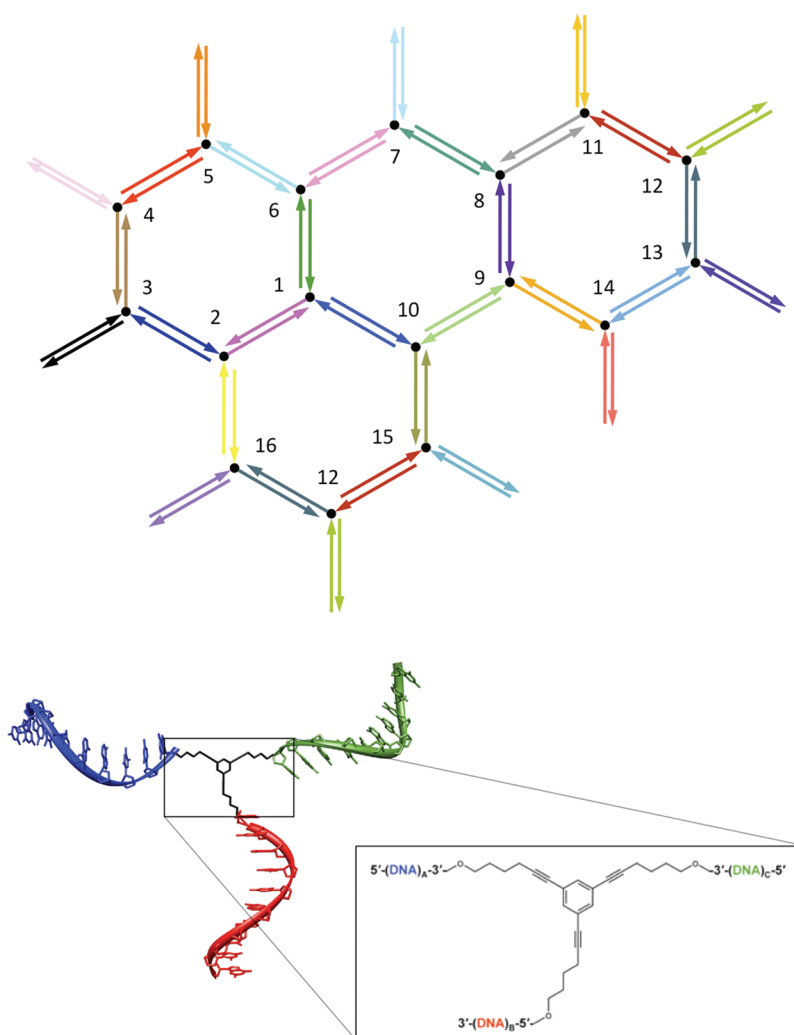


Figure 1. Top: Schematic of the four-ring DNA nanostructure. The structure consists of 16 different synthetic three-way oligonucleotides, where number 12 is used twice in the assembly to investigate the possibility of using generic building blocks. Each side corresponds to a 10 nucleotide long DNA duplex, and the color-coding indicates unique, orthogonal sequences. In addition, complementary 10-mer oligonucleotides are added to all blunt arms, making all sequences in the system double-stranded. Bottom: Schematic of the synthetic three-way oligonucleotide, with the 1,3,5-trisubstituted benzene node in the box to the right.

constructs present, and the same can be seen for the two-cell starting point, DNA-naphthalene (A). The band midway between the monomer (H) and the main band, present in all samples, corresponds to the one-cell structure or DNA-benzene, presented previously.³⁵

The other three-cell structure, DNA-phenalene, is verified in the same way as DNA-anthracene, by starting with the known two-cell structure and adding one three-way oligonucleotide in a stepwise fashion until the final three-cell structure is reached. The stepwise buildup is presented in Figure 3, starting from DNA-naphthalene (A) on the left and ending on DNA-phenalene (F). The same stepwise retardation pattern can be seen in this series of constructs, each addition of another three-way oligonucleotide yields a slower moving band in the gel. Sample E is the exception to this pattern. One would expect this construct to have similar mobility as sample D, having the same number

of three-way oligonucleotides in slightly different orientation. However, the band that would follow this logic is missing; instead, the sample displays similar bands as samples B and C, indicating that there is poor binding of the added three-way oligonucleotide. The reason for this poor binding could be some interference effect of secondary order within the specific sequence, that is, semistable hairpin-like structures of a few base pairs. This emphasizes the importance of maintaining totally double-stranded sequences within the system; even though there might be undesired side products that are energetically favorable, the free energy of these structures cannot compete with the lower free energy structures when all sequences are perfectly matched. As seen in the final DNA-phenalene sample (F), there is only one major band for large constructs visible, indicating that the designed structure is formed without major interference from

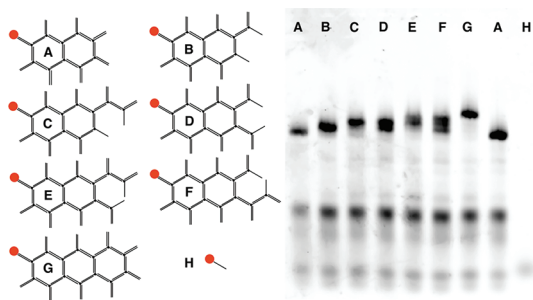


Figure 2. Stepwise formation of the three-cell construct DNA-anthracene following the schematic on the left. Analysis was done using 4.5% MetaPhor agarose gel electrophoresis. Red dots indicate the position of the Cy3 label used for visualization of product in gel scanner. For an intensity profile of the overlapping bands for sample F, see Figure S4 in Supporting Information.

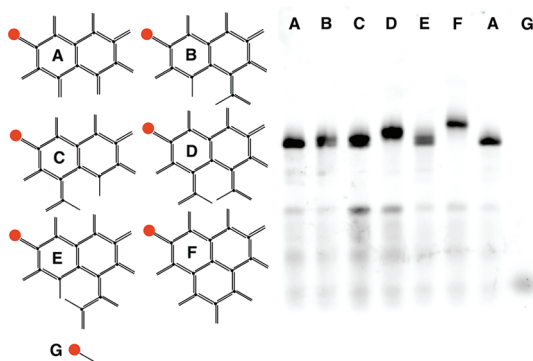


Figure 3. Stepwise formation of the three-cell construct DNA-phenalene following the schematic to the left. Analysis was made using 4.5% MetaPhor agarose gel electrophoresis. Red dots indicate the position of the Cy3 label used for visualization in gel scanner.

undesired substructures. To verify that this band corresponds to the desired DNA-phenalene and that the missing node in sample E indeed appears and binds to the structure when all building blocks are present, a gel electrophoresis experiment was performed in which a fluorophore label was positioned on this node. A band with the same migration rate thus appears in all samples irrespective of label position, verifying that the missing node of sample E coordinates when all building blocks are present (Figure S2 in Supporting Information). This observation also confirms that DNA-phenalene is indeed formed. The unstable construct is always the one with the maximum number of uncyclized flexible branches (*i.e.*, E in Figure 3, and E and F in Figure 2). As the fully cyclized versions are more stable, this must constitute a driving force for cyclization. It is possible that constructs like E (Figure 3), in which there is considerable conformational flexibility, are destabilized by the close proximity of noncomplementary single-stranded (and also double-stranded) regions that are not conformationally locked by base pairing. That is, this construct has dangling single- and double-stranded

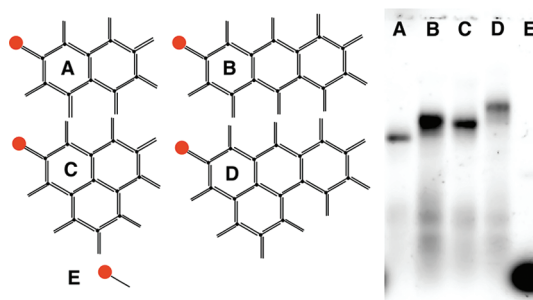


Figure 4. Four different completely ring-closed DNA nanostructures of varying size, following the schematic outline to the left of the gel. DNA-naphthalene (A), DNA-anthracene (B), DNA-phenalene (C), four-ring structure (D) and Cy3-labeled 10-mer reference (E). The gel is 4.5% MetaPhor agarose, and visualization is made using Cy3 dye as indicated by the red dot in the scheme to the left.

regions. Such regions have a high density of negative charge, and as they are not locked in place, they will be mutually repulsive. In structures such as F (Figure 3), which is fully base-paired, the specific organized binding of metal ions to the duplex (*i.e.*, to the major and minor grooves) might better be able to neutralize the high negative charge density alongside the free energy gain of hybridization.

Having demonstrated the assembly of the two different three-ring systems separately, we move forward to show the assembly of the entire four-ring structure displayed in Figure 1. Figure 4 shows the different completely ring-closed network structures that have been assembled in this study: starting from DNA-naphthalene (A), followed by the two variants of three-ring structures, DNA-anthracene (B), DNA-phenalene (C), and finally the complete four-ring network (D).

The gel in Figure 4 clearly shows that all the different structures are formed as major products of each individual assembly process. There is no substantial contribution from any specific byproduct in any of the four samples. However, there are traces of other structures in all assemblies, visible in all gels (Figures 2–4). A systematic study of yields of the desired construct for each individual assembly process can give us information about what to expect as the network grows bigger. This can be done by measuring the fluorescence intensity from the band corresponding to one specific construct, and normalizing with respect to the total intensity of the sample in one lane on the gel. The relative fluorescence intensity corresponds to the molecular ratio of that construct relative to all other constructs in the same lane, thus giving the yield (detailed information about the method in Supporting Information). This analysis was made for all ring-closed structures investigated in the current study because all gels indicate a consistent difference between fully ring-closed structures and the others. Also included in the analysis is the yield from the one-ring structure, DNA-benzene, which we presented in a previous paper.³⁵

These yield data are plotted, in Figure 5, against the number of DNA duplexes involved in forming each specific structure (n), that is, DNA-benzene ($n = 12$), DNA-naphthalene ($n = 19$), DNA-phenalene ($n = 24$), DNA-anthracene ($n = 26$), and the final four-ring network ($n = 31$). To obtain statistics, the same analysis was performed in multiple experiments carried out under the same conditions, resulting in the error bars (standard deviation) in the figure.

The yield result in Figure 5 displays as expected a monotonously decreasing trend as the number of duplexes increases, that is, the size of the network is getting larger. This behavior can be understood using a simple experimental model. Assuming that the probability of a hybridization event to occur (*i.e.*, that two complementary sequences form a DNA duplex) is uniform over the system (y_{duplex}), then the final yield (Y) is this probability raised to the power of number of events (*i.e.*, the number of duplexes in the system (n)).

$$Y = y_{\text{duplex}}^n \quad (1)$$

By fitting the unknown parameter y_{duplex} of this model to the experimental yield data presented in Figure 5, an average hybridization reaction yield of 96% was obtained ($y_{\text{duplex}} = 0.96$, $R^2 = 0.98$). To our knowledge, this is the first effort to systematically study and explain the outcome of nanofabrication through processes of DNA directed self-assembly. There are numerous reported yields of different DNA nanoassemblies based on oligonucleotides in the literature with a widespread range from 1%^{7,43} to 95%.⁴⁴ In the field of DNA origami, several of the remarkable assembled structures have impressively high yields,^{13,15,17,21} but because the assembly protocols vary, such as the amount of excess of “staple” strands compared to the M13 scaffold, it is hard to compare with assemblies based on stoichiometric (equimolar) DNA strand ratios as used here. In addition, it would be interesting to know the effect of and potential tolerance to missing staple strands on the final construct and the possibility of being able to resolve such constructs from fully assembled ones. Whatever assembly approach is chosen, the widespread of effective yields emphasizes the necessity for a fundamental investigation to gain insight into the reaction processes and facilitate the prediction of the outcome of a specific assembly design. It is highly probable that the effective yields of different assembly strategies of DNA nanofabrication may vary due to different thermodynamic and kinetic fundamentals. For example, the crystal-like DNA-tile assemblies lack the entropic effect of more flexible structures. Topology is, without doubt, a major issue when dealing with these kinds of processes, as well as the dynamics of actual assembly events. One can

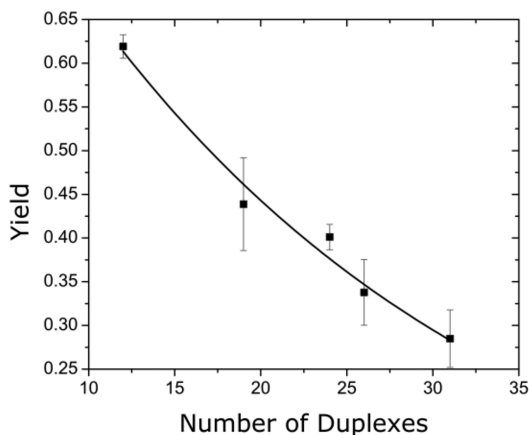


Figure 5. Yields for the ring-closed structures, plotted against the number of duplexes involved in forming one specific structure. DNA-benzene ($n = 12$), DNA-naphthalene ($n = 19$), DNA-phenalene ($n = 24$), DNA-anthracene ($n = 26$), and the final four-ring network ($n = 31$). Error bars show standard deviation for the particular structure, from repeated experiments carried out under the same conditions. The number of repeated experiments is 21 for DNA-naphthalene, 6 for DNA-anthracene, and 3 for each of DNA-benzene, DNA-phenalene, and the final four-ring network. The solid curve corresponds to the statistical yield model (eq 1).

imagine differences in topological constraints that need to be overcome when comparing fabrication in 2D and 3D, in addition to how and in what order different parts of a construct come together. There have been some studies of these aspects of DNA nanoassembly with FRET-based assays, studying the thermodynamic stability of DNA tiles⁴⁵ and furthermore the effect of multivalent interactions.⁴⁶ From our laboratory, we have reported on the thermodynamic aspects of a hexagonal DNA nanostructure as well as structural dynamics of the same system.^{47,48}

Looking at our system, based on hierarchic assembly, is there any fundamental thermodynamic reason for the yield of duplex formation to be significantly less than 100%? The equilibrium constant of this reaction can be estimated using thermodynamic data of a hexagonal DNA nanostructure presented in a previous report.⁴⁸ Assuming that an average of the six edges in that hexagon represent a characteristic 10-mer sequence in our system, the average equilibrium constant of hybridization can be estimated to approximately $1.2 \times 10^{12} \text{ M}^{-1}$ (at 4 °C). With an oligonucleotide concentration in the range of 10^{-7} M , this entails a single-stranded ratio of less than 0.5%. This is obviously not the main reason for the missing, on average, 4% of unformed DNA duplex. There must be other sources of error, and those of experimental origin are worth considering.

A potential deviation from perfect 1:1 stoichiometry is an issue close at hand. A deviation from equimolar stoichiometry may originate from volumetric uncertainty and imprecise oligonucleotide concentration

determination. The latter is based on ultraviolet absorption, using the nearest-neighbor approximation (NNA) to deduce the extinction coefficient for a given nucleobase sequence. This approximation has an inherent uncertainty of about 10%.⁴⁹ Whatever origin, an uncertainty in oligonucleotide concentration resulting in deviation from equimolar stoichiometry is an issue that needs to be addressed to determine to what extent it influences the overall yield.

If one considers an assembly of building blocks with a concentration distribution of a given variance, the way the assembly occurs may have a major effect on how the distribution influences the final product. In a sequential addition of building blocks, deviations from the average concentration will deplete the cohort of building blocks available for the desired assembly. The reason for this is that any difference between two consecutive building blocks in the chain will create alternative structures that will compete for the subsequent building blocks. Assuming no difference in adhesion between the desired structure and alternative structures, the yield of the desired structure will decrease based upon the average deviation of concentration. In this case, the situation will worsen the larger the assembly, in the same manner as our suggested yield model; that is, one may consider an average error in oligonucleotide concentration decreasing the yield of a hybridization reaction, which accumulates with increasing assembly size. In fact, the total assembly yield of a chain (Y) decreases with the average of the relative concentration differences (Δ) and system size (n). This average difference is related to the standard deviation from average concentration (σ) in the following way (details in Supporting Information).

$$Y = \left(1 - \frac{\langle \Delta \rangle}{2}\right)^n = \left(1 - \frac{\sigma}{\sqrt{\pi}}\right)^n \quad (2)$$

Applied to our system, this error would suggest that a standard deviation of 7% from 1:1 stoichiometry could explain the missing 4% in each hybridization reaction step. However, it is also important to take the effect of ring closure into account. Although ring closure is expected to increase electrostatic repulsion and decrease conformational entropy, compared to linear polymerization, it also provides a higher apparent concentration of the two ends closing the circle, in the end leading to a thermodynamically favored process, as found for a closely related single hexagon system.⁴⁸ The equilibrium model of hexagon formation from ref 48 may be expanded to a ring of any number of building blocks (n). With each oligonucleotide building block being attached to the previous one, from A to W, the last one closing the ring, the overall ring yield (Y) may be expressed in terms of an equilibrium equation involving all

building blocks, by relating the amount of formed rings (x) to the mean oligonucleotide concentration (m)

$$Y = \frac{x}{m} = \frac{(\min([A]_0, [B]_0) - x)}{([A]_0 - x)} \frac{(\min([B]_0, [C]_0) - x)}{([B]_0 - x)} \dots \frac{(\min([W]_0, [A]_0) - x)}{([W]_0 - x)} \kappa \quad (3)$$

where the concentration distribution of oligonucleotides is characterized by the lowest concentration of each stoichiometric couple (*i.e.*, A–B, B–C...W–A) limiting the ring formation (x). The dimensionless parameter κ is the ratio between equilibrium constant of ring closure and the average oligonucleotide concentration, thus, gauging the strength of ring closure. Assuming that deviations of oligonucleotide concentration from the mean value follow a normal distribution with standard deviation σ , the overall yield can be expressed as an exponential relation (see Supporting Information).

$$Y = 1 - \alpha = \kappa e^{-n/\sqrt{\pi} \left(\frac{\sigma}{\alpha} + \frac{5}{6} \left(\frac{\sigma}{\alpha} \right)^3 + \frac{43}{20} \left(\frac{\sigma}{\alpha} \right)^5 \right)} \quad (4)$$

A large κ , meaning an effective thermodynamic driving force for ring closure, could counteract the influence of stoichiometric deviations on the overall yield. However, we note that the model considers one ring only (one parameter κ), while the polycyclic system is considerably more complex with multiple branching points, for which one ring-closing parameter may be inappropriate. Anyhow, when we the gel electrophoresis results in Figures 2 and 3 are viewed, the higher yields of all ring-closed structures indeed indicate that ring closure is an important factor in the studied system that needs to be taken into account. Although the model is crude, it may give some indication how to interpret the behavior of our system. Therefore, treating the polycyclic system as rings of different sizes, it is possible to estimate the magnitude of the ring-closure efficiency if one assumes a standard deviation of 10% (given by the uncertainty in NNA) and fit this model to the experimental data in Figure 5, $\kappa = 3.6$ for this system.

Furthermore, one cannot disregard the potential interference effects of base sequence matching of secondary order. Even though the sequences are designed to be orthogonal, one should not rule out the possibility of forming undesired metastable secondary structures such as hairpins leading to a smaller effective concentration of the building blocks and even to structural arrest. Analogous to the issue with stoichiometry, this problem is expected to scale with increasing structural size in a similar way as our yield model. There could also be topological problems related to the structural design of the three-way oligonucleotide building block. Even though the system is designed to have swivel flexibility, distortions of the planar conformation might lead to some assembly problems. It would be interesting to make systematic studies with

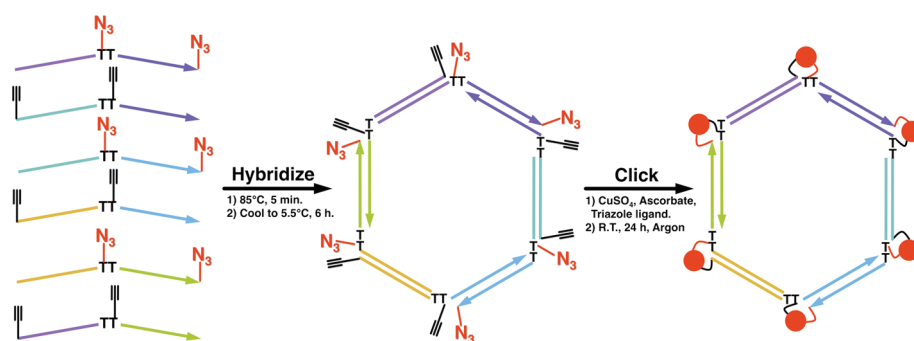


Figure 6. Reaction scheme of the site-specific click-fixation strategy of a hexagonal DNA nanostructure. Six alkyne and azide modified 22-mer oligonucleotides are hybridized to form a hexagonal construct with edge length 10 bases. The sequence of 22 nucleotides has two stretches of 10 nucleotides separated by two thymines. Each stretch of 10 bases is complementary to another sequence in the system, forming one side in the hexagon. The two thymines are unpaired and function as hinges. Upon formation of the hexagonal nanostructure, azide and alkyne modifications are situated to enable the six site-specific click reactions of the fixation step. Chemical structures of the triazole cross-links are shown in Figure S5 in Supporting Information.

varied nodes and tethers as well as DNA sequence lengths to address this.

From a nanotechnological point of view, there are two conclusions that can be drawn from our results.

- (1) A small deviation from unity for the probability of each hybridization event will have a big impact on the final result as the number of events increases. Hence, we may be rapidly reaching the limit of how large a network we can assemble in one step, with a meaningful yield.
- (2) A more general conclusion is that a one-pot reaction of multiple, simultaneous assembly events may be treated in the same way as a sequence of reaction steps in a synthesis protocol.









There are two different possible strategies to increase the yield of the final product. (i) Improve the yield of the single duplex reaction. The closer to total conversion of one hybridization reaction (y_{duplex} approaching 1), the larger the network that can be assembled in a one-step process. However, this does not solve the underlying problem that any deviation from total conversion at the single-duplex level will decrease the yield of the end product. The larger the network gets, the more substantial will be the decrease. (ii) A second solution to the one-pot issue for larger DNA nanoconstructs could be to adopt a different approach to self-assembly nanofabrication, *viz.* by instead adopting a stepwise process with purification and workup possibilities (*i.e.*, a modular buildup).

We have previously reported a click-fixation strategy for creating a larger robust building block assembled from short oligonucleotides.⁴² By incorporating azide and alkyne functionalities at specific positions, a totally covalently cross-linked DNA nanostructure can be formed using Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), that is, click chemistry.^{50,51} The model system is a hexagonal DNA nanostructure assembled from six 22-mer oligonucleotides, with an edge length

of 10 bases. Each 22-mer oligonucleotide has two 10 base sequences separated by two thymines. The stretch of 10 bases has one unique partner in the system, forming one side of the hexagon. The two thymines are unpaired, functioning as flexible hinges. Upon formation of the hexagonal nanostructure, azide and alkyne modifications are positioned to enable the six site-specific click reactions of the fixation step (reaction scheme in Figure 6; more information can be found in ref 42). We envision that such a covalently fixed nanostructure could function as the basis for assembly of larger constructs in a modular manner, a strategy that would in the future allow us to assemble very large structures by relying on prefabricated modules instead of using a considerable number of smaller building blocks assembled in one step. Solubility could be a problem when the building blocks become very large and only a small number of connectors will require rather high concentrations for good yields. However, if the size is balanced by an appropriately increased thermodynamic stability of the final assembly through a large number of hybridization sites, the assembly should be possible using dilute solutions of reactants.

When designing future modules, it is vital to understand the fixation strategy, a system where six individual click reactions occur simultaneously in a one-pot reaction. As pointed out before, the precision of these reactions is governed by the exact positioning of the alkyne and azide functionalities intended to react. This creates a system of six identical reaction sites located on a nanoscale construct (*i.e.*, a hexagon with a side length of 3.4 nm). It is possible to investigate if these reaction sites somehow interact with each other by looking at the resulting fractions of substructures formed after the click fixation process has occurred. Analogous to the case with yields of DNA nanostructures based on a one-step approach considered above, the probability that a click reaction occurs is below unity. A result of this non-ideal behavior is that instead

TABLE 1. Overview of Possible Combinations of Click Reactions (Red Dots) That Can Take Place on a Hexagonal Starting Material and the Actual Substructures Resulting from a Specific Combination

Start	No. of Click	Position	Degeneracy	Total No.	Substructures ^a
	6		1	1	H(6)
	5		6	6	L(6)
	4		6 6 3	15	5, 1 4, 2 3, 3
	3		6 6 6 2	20	4, 1, 1 3, 2, 1 3, 2, 1 2, 2, 2
	2		6 6 3	15	3, 1, 1, 1 2, 2, 1, 1 2, 2, 1, 1
	1		6	6	2, 1, 1, 1, 1
	0		1	1	1, 1, 1, 1, 1, 1

^a The numbers correspond to the size of fragments a particular click combination gives rise to, that is, the number of oligonucleotides involved. For example, the first combination of 4 successful click reactions gives rise to a 5-mer and a monomer substructure.








of one single end product, that is, one totally covalently locked DNA hexagon, there will be a distribution of various substructures corresponding to species for which one, two, or more click reactions have not taken place. The matter is further complicated by the fact that the range of substructures actually formed is not only due to the number of reactions but also dependent on where in the structure these reactions occur. Table 1 summarizes all possibilities of different numbers of click reactions, where they occur on the hexagon, and the resulting substructures, starting from a hybridized DNA hexagon. Due to symmetry, many possibilities are degenerate.

At this point of the analysis, a model can help us understand the outcome of the process. First, each of the six click reactions constitutes a Bernoulli experiment, that is, a binary option of success or failure. This means that if the reactions occur independently then the resulting outcome of the six sequential, independent events should follow a binomial distribution (eq 2). The probability, $P(k)$, of a number of successful events, k , in a number of trials, n , is given by the following equation, where p is the probability of one successful event.

$$P(k) = \binom{n}{k} p^k (1-p)^{n-k} \quad (5)$$

Applied to click-fixation n is the total of six click reactions and k is the different number of successful reactions, starting with a fully hybridized DNA nanohexagon. However, because the end result depends not only on the number of successful click reactions but also on position (as illustrated in Table 1), the analysis is somewhat more complex. The next step is therefore to evaluate each substructure separately and sum up contributions from every specific number of successful reactions that contributes to one

TABLE 2. Summary of the Contribution of Binomial Terms to the Different Substructures

Structure	Binomial Terms ^a	Values ^b
	$\frac{6}{6} \binom{6}{1} p(6)$	0.3122
	$\frac{6}{6} \binom{6}{6} p(5)$	0.4011
	$\frac{5}{6} \binom{6}{15} p(4)$	0.0716
	$\frac{4}{6} \left(\binom{6}{15} p(4) + \binom{6}{20} p(3) \right)$	0.0695
	$\frac{3}{6} \left(\binom{6}{15} p(4) + \frac{12}{20} p(3) + \frac{6}{15} p(2) \right)$	0.0633
	$\frac{2}{6} \left(\binom{6}{15} p(4) + \frac{18}{20} p(3) + \frac{18}{15} p(2) + \frac{6}{6} p(1) \right)$	0.0512
	$\frac{1}{6} \left(\binom{6}{15} p(4) + \frac{24}{20} p(3) + \frac{36}{15} p(2) + \frac{24}{6} p(1) + \frac{6}{1} p(0) \right)$	0.0311

^a The binomial terms are deduced by evaluating contributions from different origins (according to Table 1), with fractions correlated to the degeneracy of a substructure. The factors preceding the parentheses ensure every binomial term to be set to the same base (*i.e.*, parts of six). ^b The values are the binomial terms for $p = 0.82$, which applies to our experimental results for the hexagonal system.

substructure, according to Table 1. The resulting binomial terms are displayed in Table 2. The contribution from a specific number of successful click reactions to a substructure depends on the degree of degeneracy, explaining the fractional coefficient of each term. Furthermore, to be able to compare substructures with each other they have to be of the same base, that is, parts of six in this case, why there is also a factor correlated to the size of a substructure. For example, for the hexagon, there is only one, in a total of one, possibility of forming that structure, and furthermore, it is constructed of six parts out of six. This gives rise to the prefactors of the $P(6)$ terms in Table 2 which, although not important in the present example, are displayed for clarity.

An experimental parameter that has to be taken into account, if the binomial distribution model is to be used as justification for the behavior of the click-fixation system, is the presence of substructures due to incomplete hybridization (as indicated in ref 42). The result of this situation, unless a purification step is applied, is that there are DNA structures of varying sizes already at the start of the click fixation step and, notably, a decreased number of possible click-reaction sites (the linear 6-mer has 5 sites, 5-mer has 4 sites, 4-mer has 3 sites, *etc.*). Consequently, each starting template gives rise to a different binomial distribution with a different pattern of substructures, analogous to the case with the hexagonal template described in Tables 1 and 2 (corresponding tables for each starting template are given in Supporting Information). To generate an accurate representation of the system including this template spread, using the binomial model, all possible starting structures have to be taken into account. This gives an expression for each covalently cross-linked substructure in the form of a weighted

sum of binomial terms, originating from every starting template structure with its corresponding distribution (as given for the hexagonal template in Table 2; for other structures, see Supporting Information). The experimental yield data from the hybridization reaction⁴² are the coefficients used for the different substructures in the weighted sum. Starting with 27% of the hexagonal structure appearing from the hybridization reaction entails a factor of 0.27 to all the terms in Table 2 when summing the binomial distributions. Analyzing all substructures in a similar manner finally generates the binomial model for this system.

The result of this analysis is displayed in Figure 7, with the prediction of the binomial model given in black and the experimental data given in red (error bars correspond to the standard deviation of 14 identical experiments, data from ref 42). The pattern of the model corresponds well with the experimental results, within experimental error in all but two cases (4-mer and monomer). There is only one variable in this model, which is the probability of a successful event (p), that is, the probability of one click reaction occurring. This is deduced by evaluating the probability of achieving the percentage of cross-linked hexagon indicated by experimental data. Six click reactions need to occur and consequently the sixth root of the hexagonal experimental yield gives the probability of one of these events (taking the hybridization reaction into account). The experimental data of this study give a value of 82%, which is used in the presented distribution (Figure 7). Assessing this variable rather than a fitting procedure is motivated by a higher reliability for the experimental yield of the hexagonal structure compared to the monomer structure. On the polyacrylamide gels in this study, the hexagonal structure produces one distinct and easily quantified band, arising from the fact that there is only one possibility of forming this structure. The monomer structure, on the other hand, is actually six different oligonucleotides. As a consequence, the band on the gel broadens because of the sequential distribution of different monomers. Furthermore, the monomer structure behaves differently on native and denaturing gel.⁴² These phenomena make the quantification of the monomers less precise, thus the yield data of the hexagon are more reliable in comparison. An analysis procedure where the variable p was fitted to the experimental data has been investigated (data not shown), however, without improvement in presentation or understanding.

The result presented in Figure 7 indicates that the binomial model can explain the yields for the click-fixation system studied in this paper. This analysis also gives us information about possible cooperativity. Because independence of events is a prerequisite for a binomial distribution, we may conclude that the six click reactions occur without any interference with one

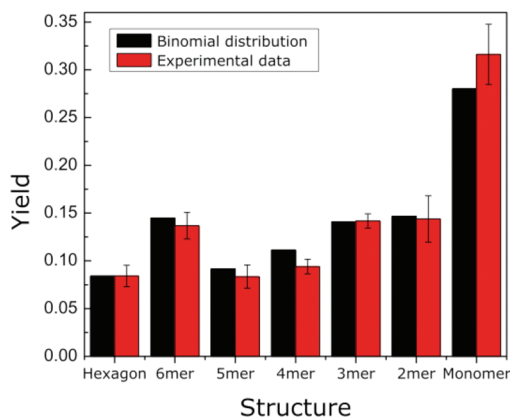


Figure 7. Yield data for click fixation of the DNA hexagon. Experimental distribution of possible substructures shown as red columns, error bars indicating standard deviation (14 samples). Experimental data taken from ref 42. Black columns show weighted sums of binomial distributions for each substructure of all possible starting materials. The probability, p (yield of one click reaction), is deduced to be 0.82 from the observed yield of totally cross-linked DNA hexagons.

another. Understanding and predicting the outcome of the click-fixation strategy could pave the way for this technology to be applied to systems of higher hierarchy using a modular approach for bottom-up nanoassembly.

CONCLUSIONS

We have demonstrated the assembly of a fully addressable nanonetwork, based on synthetic three-way branched DNA molecules. Even though the principle of total addressability relies on unique building blocks, we show that it is possible to assemble nanostructures of precise sizes containing a generic node. Furthermore, the four-ring DNA nanostructure is assembled from 28 building blocks in a one-step annealing process with a respectable yield of 28%. Importantly, we show that a simple statistical yield model can describe the limit of a one-step/one-pot assembly process for a nonperiodic system like this. The yield of DNA nanostructures with controllable size decreases exponentially with increasing structure size (*i.e.*, number of duplexes involved). Therefore, in order to open up a general route for nanofabrication based on DNA assembly, we envision an alternative future strategy that may solve the problem of decreasing yields when larger structures are desired: this is a modular buildup approach based on a click-fixation step, covalently cross-linking a DNA nanostructure and thereby creating a larger module, which is robust and importantly may be purified and used for further fabrication. Although the present yield of this module is low, less than 10%, the result defines a first step toward an alternative fabrication approach. More importantly, we show how the system of six simultaneous click reactions on a DNA nanohexagon behaves in a way that can be described by a binomial

model. Not only does this predict the outcome of the reaction but also, more importantly, tells us that the six reactions proceed independently from one another. Consequently, this model allows us to use the binomial distribution function as a probe of

cooperativity between chemical reactions on the confined template of the DNA nanostructure. Deviations from this model would indicate positive or negative chemical interference, a scientific path we will follow in future studies.

METHODS

DNA Sequence Design. The system follows the same principals as in our previous work.^{35,36} The three-way oligonucleotides, serving as basis for the assembly, are synthesized using a 1,3,5-trisubstituted-benzene as node. The D_{3h} -symmetric node gives rise to the hexagonal geometry of the unit cell. However, a nonrepetitive network demands unique sequences on all three positions, which can be achieved by incorporating suitably chosen orthogonal protection groups in the synthetic process. This enables not only the necessary freedom in base sequence design but also freedom in directionality of the strands. In the system of this study, all nodes are connected to the 5' end of one strand and the 3' end of the two other strands (Figure 1). The reason for this geometry is simplicity and optimization of the reaction yields in the solid-phase synthesis; this configuration is consistent with the use of conventional 5'-dimethoxytritylnucleoside-3'-phosphoramidite monomers in trigonal oligonucleotide assembly. Experimental details can be found in previous work.^{35,36} Each side in the system is a unique sequence of 10 nucleotides, and every 10-mer sequence is designed to be complementary to only one other sequence in the system and orthogonal to all others. The criterion of orthogonality between two sequences is that no more than four bases may match for any alignment of two strands. Furthermore, to enhance the thermodynamic stability of the system, the number of CG bases in any 10-mer sequence is set to be at least four. All three-way oligonucleotides in the system are unique apart from one (12 in Figure 1), which is used twice in the nanoconstruct. This assesses the possibility of using generic nodes in the system and how this might influence the formation of the desired construct.

Self-Assembly Process. Every oligonucleotide was set to 2 μ M in phosphate buffer (pH 7.5; $[\text{Na}^+] = 200$ mM; $[\text{PO}_4^{3-}] = 109$ mM) using the DNA absorbance at 260 nm, with the extinction coefficients at 260 nm calculated by the nearest-neighbor approximation (NNA). To assemble a specific construct, the oligonucleotides involved were mixed in equimolar amounts (typically 1.5 μ L per oligonucleotide) and hybridized by heating to 90 °C for 5 min and allowing to cool to room temperature overnight (typically 15 h) by turning off the heating block (Grant Inst.). The samples were then put on ice until further analysis.

Gel Electrophoresis. All samples were analyzed using 4.5% (w/w) MetaPhor agarose (Cambrex) with phosphate buffer (pH 7.5; $[\text{Na}^+] = 200$ mM) as running buffer. Ficoll 400 (Sigma-Aldrich) was added to each sample as a loading agent, giving a concentration of 5% (w/w). The applied voltage was set to give a field strength of 3.5 V/cm, typically 67 V. The run time of electrophoresis was 4 h. Circulation of the buffer through a heat exchange system was performed to keep the temperature of the system below 4 °C throughout the experiment.

Sample Analysis. After electrophoresis, the gels were visualized using a Typhoon 9410 (GE Healthcare) using appropriate laser excitation and emission filter for each fluorophore. Cy3 was excited with a 532 nm laser and emission collected through a 580 nm band-pass filter. Yield estimations were done by quantification of emission intensities from each band correlated to the total intensity of the sample using ImageQuant TL software (GE Healthcare).

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Supporting Information Available: DNA sequences of oligonucleotides; analysis of the binomial distributions, starting from templates of different sizes and yield error analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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