A New Fixation Strategy for Addressable Nano-network Building Blocks

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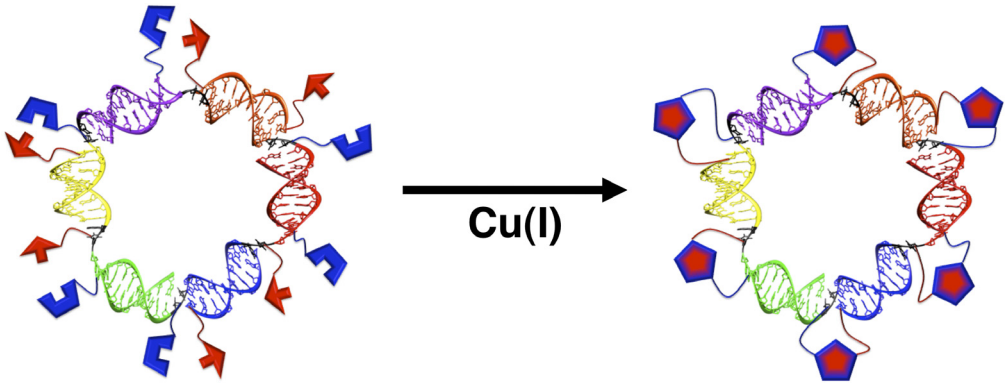
Abstract

Rapid controlled self-assembly makes DNA ideal for building nanostructures. A problem using hybridized intermediates in hierarchic assembly is their thermodynamic lability. We demonstrate a click-fixation technology by which robust hexagonal DNA modules can be made. This principle is applicable to a wide variety of DNA nanoconstructs.



We report the successful covalent cross-linking of a six-membered DNA circularized nanostructure using click chemistry, taking the first step towards modular build-up of fixated larger networks for use as templates in nanotechnological applications.[1] The inherent ability of DNA to form double helices by self-assembly through Watson–Crick base pairing has been successfully demonstrated in various contexts for creating advanced supramolecular structures.[2-7] The assembly and stability of such nanostructures relies on precise hydrogen bonding as well as base stacking and the non-covalent bonding makes these systems delicate, potentially unstable and unsuitable for use as building blocks for larger systems. Ligase enzymes may be used to join together ends of DNA strands[4] but this is restricted to sealing nicks in linear sequences, which limits versatility in DNA nanostructure design. Moreover, the enzymatic action of ligases is unlikely to work efficiently with complex highly constrained dense nanoconstructs. Therefore, finding chemical strategies for fixation of self-assembled DNA nanostructures that can be used as building blocks is important. The copper(I)-catalyzed cycloaddition between an azide and a terminal alkyne forming a triazole (CuAAC reaction) is an attractive reaction within the concept of click chemistry,[8-10] having potential use in chemical nanotechnology. Inspired by Kumar *et al*.[11] we have extended click DNA ligation to the irreversible locking of DNA structures, a concept that has not hitherto been used in nanotechnology. The developed technology is a versatile fixation tool enabling multiple simultaneous interstrand cross-linking at

precise positions within a complex DNA nanoconstruct. Since our purified cross-linked DNA modules can be further used in assembling functional supra-structures, the fixation and purification strategy presented here provides a great advantage in nanotechnologies based on self-assembly of non-periodic structures; the structures can be made more robust, which will be a prerequisite for many applications.

The hexagonal DNA nanostructure is based on the design by Tumpane *et al.*[6] Six 22-mer oligonucleotides are designed to hybridize to form a hexagon, each side 10 bases long. The sequences are chosen to have unique partners within the construct. Furthermore, all sides are bridged by two unpaired thymines (T; see Figure 1), forming the hinges in the final hexagonal construct (sequences in ESI). Cross-linking of the hexagonal nanostructure is done *via* azide and alkyne modifications on the six oligonucleotides. To covalently lock the complete structure all six strands have to be linked together, requiring six individual reactions sites. Three oligonucleotides are modified with two alkynes, one at the 5′-position and the other internally at the first unpaired thymine of the TT-hinge. The other three oligonucleotides are modified with two azides, one at the 3′-position and the second internally at the second unpaired thymine of the TT-hinge. This design enables all six oligonucleotides to be sequentially cross-linked creating a covalently locked nanostructure. A schematic of the process is presented in Figure 1. All six click reactions are performed simultaneously on the annealed hexagonal DNA nanostructure (reaction conditions in ESI). The positioning of the azide and alkyne modifications on the oligonucleotides ensures that only the desired cross-linking takes place. Furthermore, the kinetics of the reaction is maximized by positioning the reactants close together in space. [12]

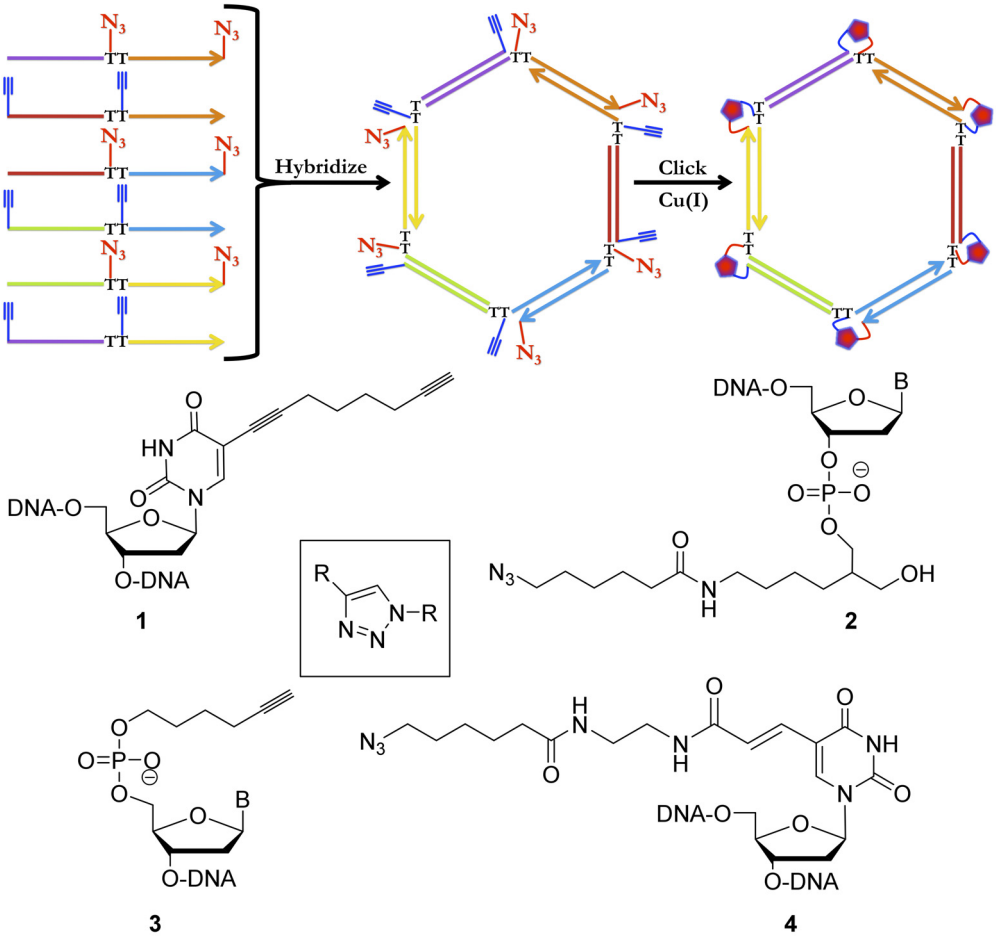


Figure 1 (a) Six azide and alkyne modified 22-mer oligonucleotides are hybridized to form a hexagonal nanostructure. Color-coding shows strands that are complementary. The nanostructure is then locked covalently by six simultaneous click reactions in which azide and alkyne form a triazole in presence of Cu(I). (b). Chemical structures of alkynes and azides in DNA. Alkyne **1** is paired with azide **2** and alkyne **3** with azide **4**. The resultant triazole linkage is shown in the box.

Figure 2 shows the result after the click reaction is performed on the hexagonal nanostructure, analyzed with denaturing polyacrylamide gel electrophoresis (PAGE). Lane 2 is the crude reaction mixture containing a distribution of the different sub-structures, from monomer (bottom) to the desired hexagon (top). Comparing this with lane 1, containing the same hexagonal DNA sample without addition of Cu(I), it is evident that the click reaction has successfully created a covalently cross-linked DNA nanostructure. The different substructures in lane 2 correspond to all possible structures formed when one or more of the six reactions fail (yields of individual structures are given in Figure 2). It should be noted that the hybridization step does not produce only the hexagon, but also a distribution of the different substructures (see ESI). Based on the amounts of non-covalent hexagons available for the click reaction (27%), the yield of the fixated hexagon is 31% (dividing 8.4% by 0.27). Considering that six individual reactions must take place for the hexagon to be completely cross-linked, the yield of each click reaction is estimated at about 82%.[13]

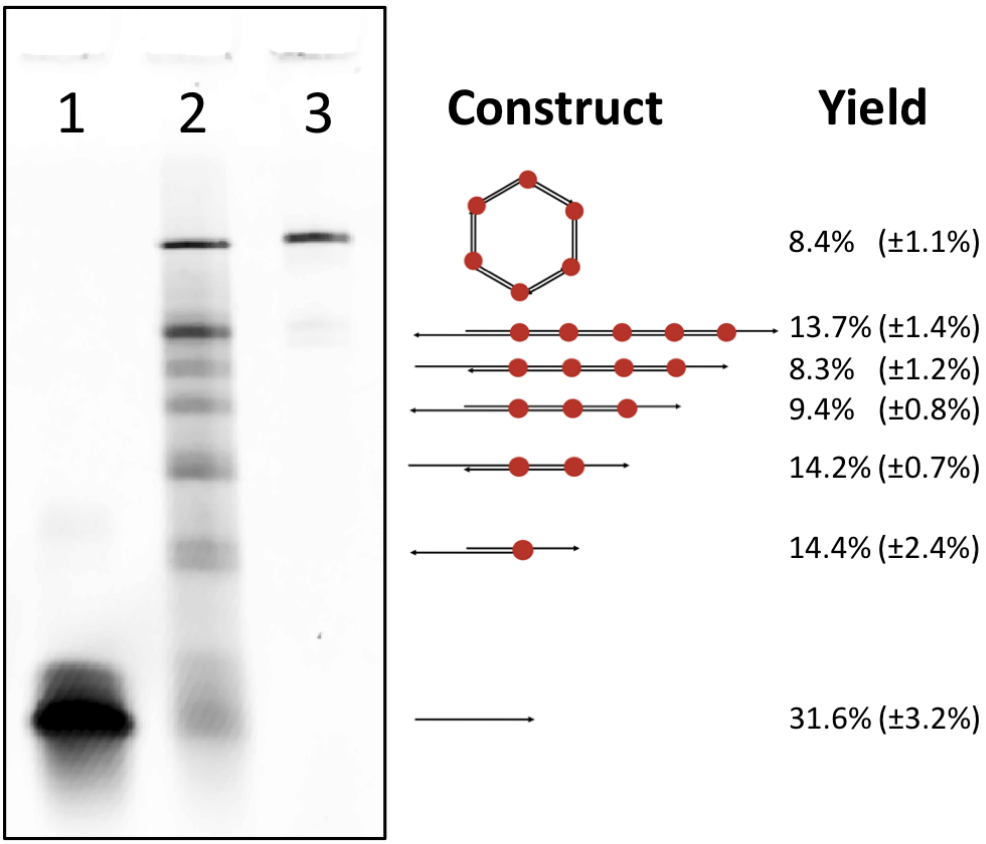


Figure 2 The click reaction analyzed with 10% denaturing PAGE. Control sample without Cu(I) (lane 1), crude click reaction sample (lane 2) and the cross-linked hexagon extracted from crude reaction mixture (lane 3). The sketches indicate the constructs responsible for the corresponding bands and the number of successful click reactions involved. Yields calculated from 14 reactions proceeding under identical conditions.

Figure 3 shows the step-wise build-up of the hexagon, each lane showing a specific sub-structure, from monomer (lane 1), dimer (lane 2) to hexagon (lane 7). The linear hexamer (lane 6) and the hexagon (lane 7) have the same number of bases and only differ in geometry; the two-dimensional extension of the hexagonal structure markedly attenuates the electrophoretic mobility. The crude reaction mixture (lane 8) obviously contains a distribution of the different sub-structures, creating a ladder for the system. All samples in lanes 1–7 have been extracted from a crude sample run on a separate gel (purification procedure described in ESI). All sub-structures (lanes 1–6) can be formed with different combinations of the oligonucleotides in the system leading to broader bands in the gel. In contrast, the hexagonal nanostructure is a unique construct, giving rise to a distinct band (lane 7). This cross-linked DNA nanostructure formed from the click reaction is robust and maintains its composition even after freeze-drying and addition of denaturing agents such as formamide.

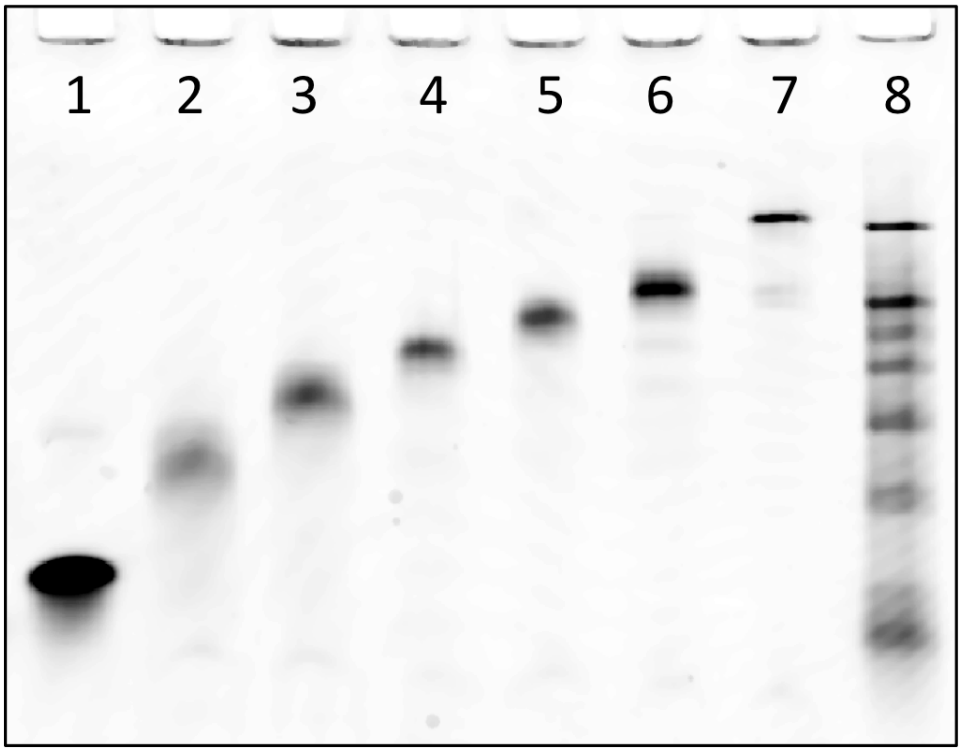


Figure 3 Step-wise build-up of the hexagon analyzed with 10% denaturing PAGE. Monomer (lane 1), dimer (lane 2), trimer (lane 3), tetramer (lane 4), pentamer (lane 5), hexamer (lane 6), hexagon (lane 7) and crude reaction mixture (lane 8). Samples 1–7 have been extracted from a crude reaction mixture.

By designing an alternative structure lacking one fixation site, the ring opening of the hexagonal nanostructure can be investigated further. Replacing two adjacent 22-mer oligonucleotides with corresponding sequences lacking one azide and alkyne modification, respectively, creates a construct that can hybridize into the ring-closed hexagon but with just five click reaction sites. This construct will therefore open under denaturing conditions forming a linear hexamer. To monitor this transition a fluorophore (FAM) and a quencher (dabcyl) were introduced at the hexagon side of interest, one on either strand, forming a donor–acceptor FRET-pair. Upon melting of the side lacking fixation, the distance between donor and acceptor will increase whereby the donor emission will increase due to reduced energy transfer to the acceptor. Figure 4 illustrates the difference between the totally fixated construct with all six click reaction sites and the alternative construct lacking one fixation site. The totally fixated construct obviously keeps its hexagonal integrity under denaturing conditions, in contrast to the construct with only five fixation sites, whose ring opens under denaturing conditions forming the linear hexamer. This can be seen by comparing the mobility of the constructs in native and denaturing PAGE (Figure 4 a & b, respectively). Under native conditions the two constructs move with the same mobility because they are both hexagonal, but in denaturing PAGE the construct lacking one fixation site displays much higher mobility. This demonstrates the great influence of geometric shape of the constructs on electrophoretic mobility. Two constructs with the same number of bases but with different geometries, one hexagonal and the other linear, exhibit a large difference in mobility. Because of the incorporation of a FRET-pair at the side of interest it is possible to monitor the denaturing process by fluorescence spectroscopy. The emission of FAM was measured before and after addition of denaturing formamide, with normalized peak intensity presented in Figure 4c. There is a drastic increase in donor emission for the construct with five fixation sites when subjected to chemical melting, evidencing ring-opening of the hexagonal structure. By contrast, the totally closed construct keeps its hexagonal integrity, consequently the distance between the FRET-pair is unchanged and consequently no significant increase in donor emission is observed. Obviously, it is possible to control this process with temperature instead of denaturing agent. By slowly heating the samples and continuously measuring the emission, a melting temperature can be obtained. The construct lacking one fixation site displayed a characteristic increase in emission and a melting temperature of 38 °C (Fig. S4 in ESI). Placing this in the context of an earlier study on the thermodynamic properties of a similar system,[14] the melting temperature is about 3 °C higher than an equivalent base sequence.

This increase in melting temperature is not surprising bearing in mind the intramolecular entropic effect, which will stabilize hybridization. As expected the totally closed construct displays no emission increase in the temperature range studied; only a slight decrease in emission originating from the temperature dependence in quantum yield of the fluorophore was detected (Figure. S4 in ESI).

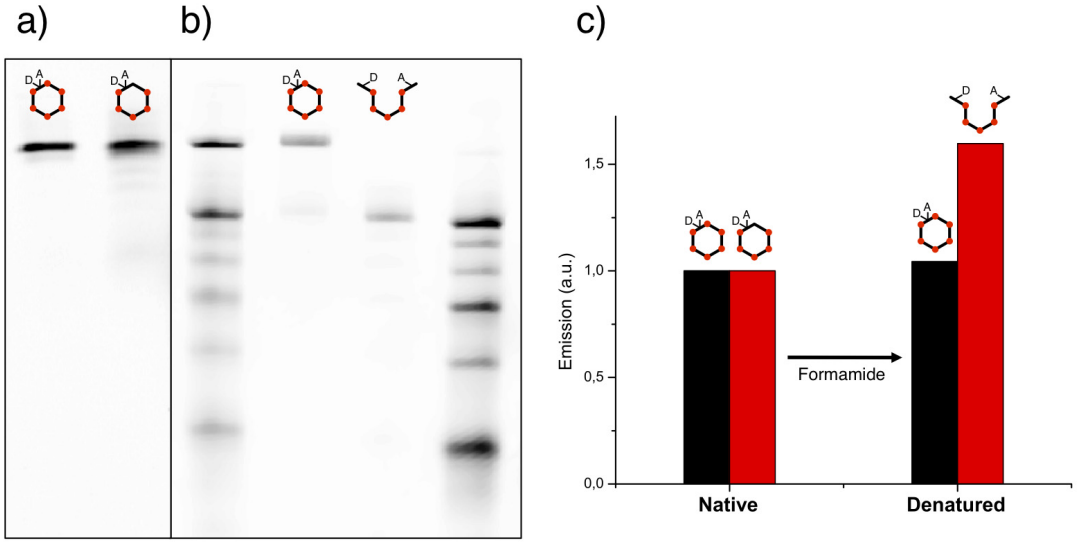


Figure 4 (a) Native 10% PAGE. Left lane contains purified structure with six click reactions forming a covalently closed hexagon. Right lane contains purified structure with five fixation sites, rehybridized to form the hexagon. (b) Denaturing 10% PAGE. From left to right: crude reaction mixture with six fixation sites containing all substructures; purified structure with six fixation sites forming a covalently closed hexagon; purified structure with five fixation sites, denatured into linear hexamer; crude reaction mixture with five fixation sites containing substructures from monomer to linear hexamer. (c) Emission peak intensity of FAM (D) in samples of purified construct with six (black bars) and five fixation sites (red bars), respectively. Upon addition of a denaturing agent, formamide, the sample with just five fixation sites ring-opens, whereby the FRET-pair, FAM (D) and dabcyl (A), separate, and emission intensity of FAM increases. On the other hand, the sample with all six sites fixated is completely covalently locked and will remain ring-closed, thus no increase of FAM emission.

In conclusion, we have used click chemistry as a chemical ligation tool to construct a DNA nanostructure, which is covalently cross-linked. Our click fixation technology was shown to be able to achieve six individual reactions simultaneously. Secondly, we have demonstrated that one may extract the desired nanostructure from a crude reaction mixture, providing the possibility of large-scale production. This is the first step towards a modular build-up of larger nano-networks, based on hexagonal unit-cells in a system with synthetic three-way nodes.[7] Working with the smallest practical units of DNA (each hexagon edge being one turn of a double helix) the system is truly fit for molecular nanotechnology.

The award to B.N. from King Abdullah University of Science and Technology (KAUST) is acknowledged.

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Footnote

Electronic supplementary information (ESI) available: DNA sequences, synthesis of modifications, hybridization reaction, melting studies and reaction conditions. See DOI: 10.1039/c001513j

Supplementary Information

A New Fixation Strategy for Addressable Nano-Network Building Blocks

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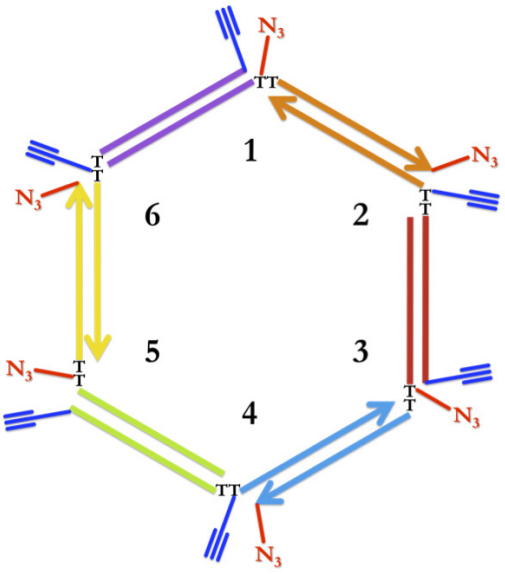

1.	5' G ATT AGC GTC $T^{Z}T$ CGA TGG TAT C^{Z} 3'
2.	5' C^{K} TGT AGA GCC TT^{K} GAT ACC ATC G 3'
3.	5' G GCT CTA CAG $T^{Z}T$ GAG GAG GAT G^{Z} 3'
4.	5' G^{K} TAT GTA TGG TT^{K} CAT CCT CCT C 3'
5.	5' C CAT ACA TAC $T^{Z}T$ CCA CAG CAT C^{Z} 3'
6.	5' G^{K} ACG CTA ATC TT^{K} GAT GCT GTG G 3'
1F.	5' G ATT AGC GTC $T^{Z}T$ CGA T^{F}GG TAT C^{Z} 3'
2D.	5' C^{K} TGT AGA GCC TT^{K} GAT ACC AT^{D}C G 3'
1F*.	5' G ATT AGC GTC $T^{Z}T$ CGA T^{F}GG TAT C 3'
2D*.	5' C^{K} TGT AGA GCC TT GAT ACC AT^{D}C G 3'

Figure S1. Base sequences of the oligonucleotides in the system. The color-coding indicates complementary sequences used to build up the hexagonal DNA nanostructure. Superscript Z and K represent azide and alkyne modifications, respectively. Superscript F and D represent FAM and dabcyl modifications, respectively. 5'-alkyne reacts with internal azide and internal alkyne reacts with 3'-azide. See Figure S5 below for precise chemical structures.

DNA sequences

The sequence of the modified 22mer oligonucleotides used in this paper can be seen in Figure S1. Two 10mer sequences on each oligonucleotide are designed to be complementary to only one other sequence in the system, indicated by the color-coding in the figure above. The sequences are designed to ring-close, forming a hexagonal nanostructure with each side being ten bases long. Two unpaired thymines bridge every 10mer sequence creating the necessary hinges in the system. The superscript Z (sequence 1, 3, 5) and K (sequence 2, 4, 6) represent the azide and alkyne modifications respectively. The oligonucleotide 1F* are similar to 1F but lack 3'-azide; 2D* are similar to 2D but lack internal alkyne. These oligonucleotides are used to form the hexagonal construct with only five fixation sites, subject to ring opening. Superscript F and D represent FAM and dabcyl modifications, respectively. The chromophores act as a FRET-pair, FAM being the donor and dabcyl the acceptor. In the experiments, all oligonucleotides are set to 2 μM using the absorbance at 260 nm. The extinction coefficients at 260 nm were calculated by the nearest-neighbor approximation (NNA).

Hybridization reaction

The hybridization reaction was performed by mixing equimolar amounts of all six 22mers in 200 mM NaCl, heating to 85°C for 5 min and slowly cooling to 5.5°C over 6 hours with a linear gradient. Different samples containing sub-structures from monomer to pentamer were also prepared (see Figure S2) to show the step-wise build-up of the hexagon. The build-up was done starting with sequence 1 as monomer (lane 1) followed by sequence 1 and 2 as dimer (lane 2), sequence 1, 2 and 3 as trimer (lane 3), and so on ending with all sequences in one sample creating the hexagon (lane 6). Samples were analyzed using native 10% PAGE (Ready Gels, Bio-Rad) on a Mini-protean 3 Cell system (Bio-Rad). Ficoll 400 (Sigma-Aldrich) was added to each sample as a loading agent, giving a concentration of 5% (w/w). The PAGE was run in 1xTBE at 70 V, giving a field strength of approximately 8 V/cm, for 2 h 45 min. Circulation of the buffer through a heat exchange system was performed to keep the temperature constant at 4°C. The gel was post-stained with SybrGold (Invitrogen) for 5 min and visualized using a Typhoon 9410 (GE Healthcare) with excitation 488 nm and 520 nm band pass filter. Figure S2 show that the hexagon is the major product when all sequences are mixed (lane 6). The faint band above the strong hexagon band in lane 6 most likely corresponds to the ring-closed 12mer (dodecahedron). The two fastest moving bands in lane 4 to 6 both correspond to monomers. The difference in gel-mobility is sequence-dependent

which can be seen in Figure S3 where each 22mer sequence is run separately. In Figure S3, lane 1 contains a hexagon sample followed by the three azide modified monomers: sequence 1 (lane 4), sequence 3 (lane 2) and sequence 5 (lane 3). The three alkyne modified monomers: sequence 2, 4 and 6 are in lane 5, 6 and 7, respectively.

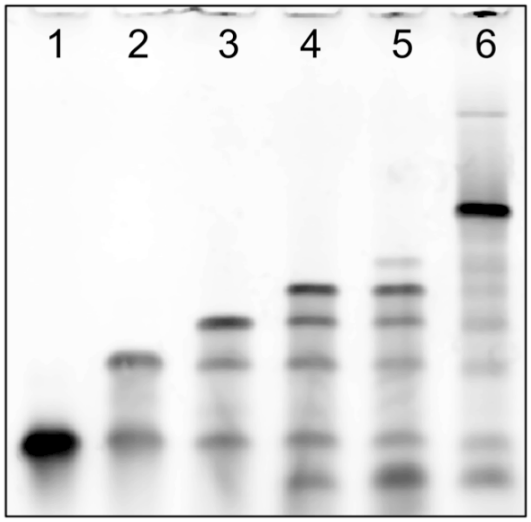

Figure S2. The hybridization reaction analyzed with native 10% PAGE. (Lane 1) Monomer (sequence 1), (lane 2) dimer (sequences 1 & 2), (lane 3) trimer (sequences 1, 2 & 3), (lane 4) tetramer (sequences 1, 2, 3 & 4), (lane 5) pentamer (sequences 1, 2, 3, 4 & 5) and (lane 6) hexagon (all sequences).

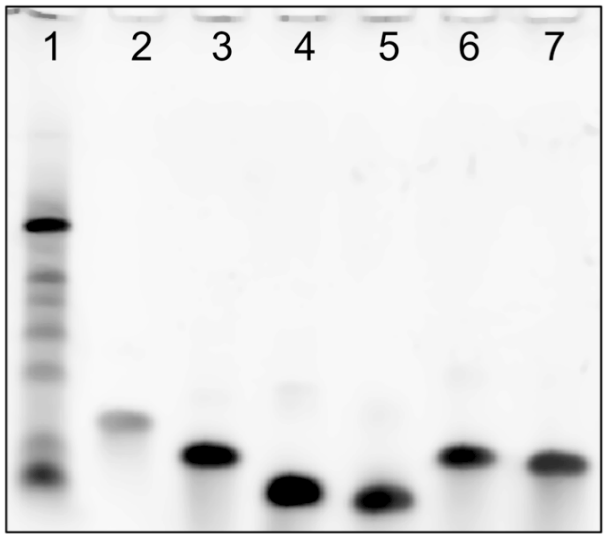

Figure S3. 22mer sequences analyzed separately with native 10% PAGE. (Lane 1) Hexagon sample, (lane 2) sequence 3, (lane 3) sequence 5, (lane 4), sequence 1, (lane 5) sequence 2, (lane 6) sequence 4 and (lane 7) sequence 6.

Looking more closely at the result of the hybridization reaction in Figure S2, the fraction of the different sub-structures where analyzed when all sequences were mixed (lane 6, Fig. S2). To get better statistics of the yield of the hexagon, several identical samples (n=8) were prepared and analyzed with PAGE using the same conditions as described above. The quantification was done using the emission intensity from individual bands analyzed in ImageQuant TL (GE Healthcare).

Structure	Yield
Dodecahedron (12mer)	2.7% (±0.6%)
Hexagon (6mer)	26.9% (±4.0%)
6mer	9.3% (±1.1%)
5mer	12.4% (±1.1%)
4mer	10.0% (±0.8%)
3mer	10.1% (±0.8%)
2mer	7.6% (±1.3%)
Monomer2	7.7% (±1.7%)
Monomer1	13.3% (±2.2%)

The analysis of the hybridization reaction was preformed at 4°C, which is well below the melting temperature of the construct (T_m = 31 °C, ref 9 in main text). Previous studies on the effect of ionic strength on T_m indicated that it is possible to raise the melting temperature of the construct by 8°C using a Na^+ concentration of 500 mM. At higher salt concentration, very little increase in T_m is observed. A major risk in raising the salt concentration higher is that mismatched duplexes will be stabilised and incorrect constructs may form. Actually, the resistance of the linear DNA constructs to bending is a more likely source of the moderate yield of the cyclic nanoconstruct, as implied by the fact that the major impurity is indeed the linear construct. The monomer units left over after the hybridization reaction points towards difficulties in estimating exact 1:1 stoichiometry for the participating DNA strands when preparing the hybridized nanoconstruct.

Click reaction

Typically CuSO4•5H2O (0.45µl 10mM) (Sigma-Aldrich), tris-hydroxypropyl triazole ligand[1] (0.7µl 45mM) and Sodium (L-)Ascorbate (0.9µl 50mM) (Sigma-Aldrich) were mixed before added to a degassed (Argon 5 min) solution of hybridized oligonucleotides (90µl 2µM), see above for details. The reaction mixture was then kept under Argon in room temperature for 24 hours before reagents were removed using a disposable NAP-5 column (GE Healthcare). The eluted volume was freeze-dried to remove excess water and redissolved in MilliQ (MilliPore) water (typically 20 µl) to appropriate concentration for analysis with denaturing PAGE. Prior to loading the samples to the gel, formamide was added to equal volume of sample. The samples were then heated to 90°C for 5 min, then immediately set on ice. This was done to ensure complete denaturation of the DNA samples. The protocol for PAGE was the same as described above for the hybridization reaction, apart from the use of denaturing 10% PAGE (Ready Gels, Bio-Rad).

Extraction procedure

After staining gels with SybrGold (Invitrogen), they were visualized on a UV-table. Specific bands containing desired DNA constructs were then cut out from the gel and placed in a 1.5 mL tube (Eppendorf). After addition of 1 mL MilliQ the sample was vortex-mixed and kept at 37°C, typically for 24 h. Quick vortex-mixing was followed by centrifugation, after which the supernatant was removed. The supernatant was desalted using a disposable NAP-10 column (GE Healthcare) and the eluted volume was freeze-dried and resolved in MilliQ (MilliPore) water (typically 20 µl) to appropriate concentration.

Emission spectroscopy

Two hexagonal samples were prepared as described above: hybridized, click fixated, extracted from gel and re-dissolved in 200 mM NaCl; one construct with all six fixation sites and the other with only five fixation sites, respectively. Both hexagonal samples contained the FRET-pair FAM and dabcyl. Prior to spectroscopic experiments, the samples were rehybridized to form the ring-closed construct. Emission spectra were recorded on a SPEX Fluorolog τ2 spectrofluorimeter. FAM was excited at 490 nm and the emission recorded between 495 nm and 750 nm. The denaturing agent formamide was added to both samples (20 µl in 60 µl original sample volume) and emission spectra were recorded again. The peak intensities (523 nm) were compared after the dilution effect had been taken into account.

Fluorescence melting curves were recorded on a Cary Eclipse (Varian) equipped with a multicell temperature block. The temperature range was 6°C to 65°C with a rate of 2°C/min. Samples were excited at 490 nm and the emission measured at 515 nm. The emission was measured at a temperature interval of 1°C. The melting curves of the hexagonal construct with five fixation sites and the totally fixated hexagon are presented in Figure S4. The increase in emission of the first construct corresponds to the separation of the FRET-pair upon ring-opening of the system (Figure S4, left), with a T_m of 38°C. The almost linear decrease in emission of the totally fixated hexagon corresponds to the change in quantum yield of the fluorophore with higher temperature (Figure S4, right).

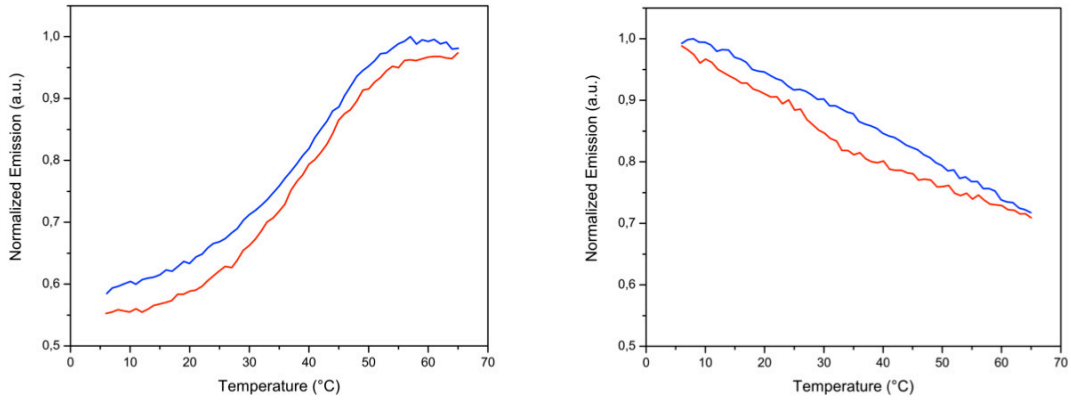

Figure S4. Fluorescence melting curves of the hexagonal construct with five fixation sites (left) and the corresponding construct with six fixation sites (right).

Oligonucleotide synthesis and purification

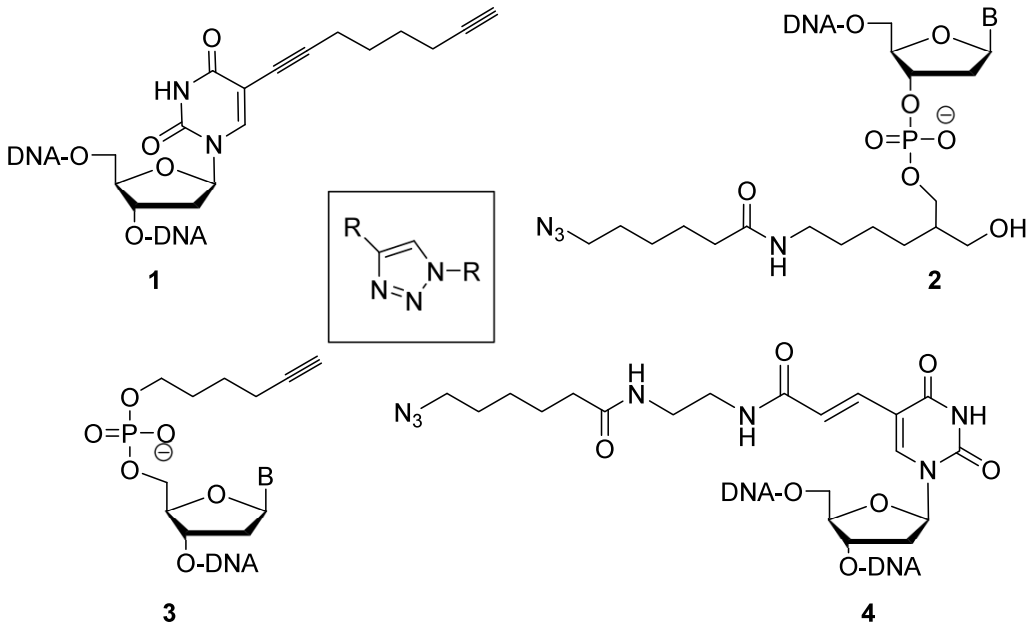

Figure S5. Chemical structures of alkynes and azides used in the formation of the closed hexagon in DNA. Alkyne 1 reacts with azide 2 and alkyne 3 reacts with azide 4. The resultant triazole linkage shown in the box.

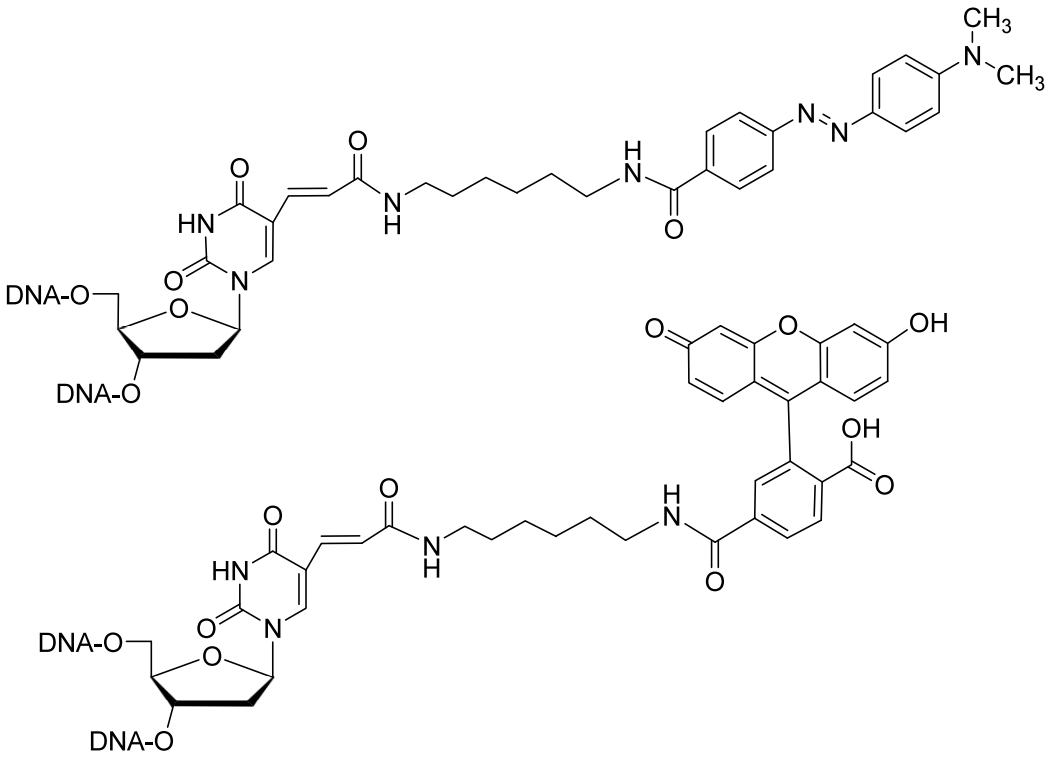

Figure S6. Fluorescein dT (bottom) and dabcyl dT (top) incorporated into DNA.

Oligonucleotide synthesis was carried out on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping and iodine oxidation. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 35 s, and this was extended to 10 min for the non-standard phosphoramidite monomers of hexynol,[2] fluorescein dT, amino C2 dT and dabcyl dT. The fluorescein dT, amino C2 dT and dabcyl dT phosphoramidite monomers and amino C7 cpg were purchased from Link Technologies Ltd. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring and in all cases were >98.0%. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 ^{o}C. Amino-modified oligonucleotides were labelled with azidohexanoic acid NHS ester as previously described.[2, 3]

Purification of oligonucleotides was carried out by reversed-phase HPLC on a Gilson system using a Brownlee Aquapore column (C8, 8 mm x 250 mm, 300 Å pore) with a gradient of acetonitrile in ammonium acetate increasing from 0% to 50% buffer B over 30 min with a flow rate of 4 mL/min (buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate with 50% acetonitrile pH 7.0). Elution of oligonucleotides was monitored

by ultraviolet absorbtion at 305 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare) according to the manufacturer's instructions.

References

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