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"Printing" DNA Strand Patterns on Small Molecules with Control of Valency, Directionality and Sequence

Tuan Trinh,[a] Daniel Saliba, [a] Chenyi Liao,[b] Donatien de Rochambeau,[a] Alexander Lee Prinzen,[a] Jianing Li, [b] and Hanadi F. Sleiman[a]*

Abstract: *The incorporation of synthetic molecules as corner units in DNA structures has been of interest over the last two decades. In this work, we present a facile method to generate branched small molecule-DNA hybrids with controllable valency, different sequences and directionalities (5'-3') using a "printing" process from a simple 3-way junction structure. We also show that the DNA-imprinted small molecule can be extended asymmetrically using polymerase chain reaction and can be replicated chemically. This strategy provides opportunities to achieve new structural motifs in DNA nanotechnology and introduce new functionalities to DNA nanostructures.*

DNA base-pairing is one of the most reliable and programmable interactions in nature. These remarkable properties make this molecule a unique template to finely organize and control matter at the nanoscale.[1] Most current approaches, such as DNA tile assembly or DNA origami, use unmodified DNA strands to guide the assembly and rely on crossover motifs to create two- and three-dimensional structures.[2] An attractive complementary approach involves the use of branched small molecule-DNA hybrids, composed of multiple DNA strands covalently attached to a small molecule core, to replace crossover motifs as building blocks for DNA nanostructures.[3] Using organic vertices with specific geometries in DNA nanostructures can significantly reduce the number of strands required, greatly influence assembly outcome, and increase DNA stability and assembly cooperativity.[3a,4][5] Moreover, by simply changing the small molecule core in the building block, new geometries and functionalities can be added.[6] Branched DNA structures can be used to enhance hydrogel[7] and nanoparticle formation,[8] DNA metallization,[9] DNA network formation[10] and DNA crystallization.[11] However, despite their significant promise, branched DNA hybrid structures have not been extensively examined as building blocks for DNA nanotechnology, in comparison to DNA origami or tile assembly. This is largely due to synthetic challenges in generating these structures. Branched DNA-small molecules with identical arms have been generated using solid- or solution-phase synthesis.[3d, 12][13][14] Over the past years, our group [3b] and others [3d,15] have attached two different DNA strands on synthetic vertices using solid-phase phosphoramidite chemistry, and used them in various applications. However, the attachment of three or more different DNA strands on a molecular core requires the laborious synthesis of molecules with multiple orthogonal protecting groups, followed by sequential build-up of DNA strands, and suffers from low yield and tedious purification. Moreover, controlling DNA (5'-3') directionality using previous methods is difficult,[16] and is limited to costly and low-yielding reverse amidites.[4e] To the best of our knowledge, there is no previous report of a facile synthetic methodology to covalently link different DNA strands to small molecules with controllable valency, sequence and directionality.

Our group has recently introduced the concept of DNA "printing", which is the covalent transfer of specific patterns of DNA strands from a DNA nanostructure onto other materials, such as gold nanoparticles and polymers.[17] Herein, we report a simple method to covalently "print" different DNA strands from a self-assembled 3-way junction to a small molecule core using copper-catalyzed alkyne-azide "click" chemistry (Fig. **1a**). DNA-templated synthesis has evolved considerably as a powerful method to control and enable reactivity of synthetic molecules.[18] Our proposed method relies on using a DNA template to ensure high effective concentration of reactive units in the middle of the junction, hence facilitating conjugation reactions. As a proof of concept, we transfer three and four different DNA sequences on triazide- and tetraazide-functionalized cores, respectively (Fig. **1**,**2**). We demonstrate that the isolated products have exactly pre-determined numbers and DNA sequences and can be tuned with respect to DNA directionality and length. Each arm of the asymmetric branched DNA-small molecule product can be elongated separately to different lengths in high yield using the polymerase chain reaction (PCR) (Fig. **4**). We then use branched DNA structures as templates for high-yielding chemical replication to generate 'daughter' branched structures, thus increasing the scalability of this approach (Fig. **5**). Our strategy offers several advantages compared to the previous methods: **1)** It uses commercially available starting materials and simple, symmetrically modified small molecule cores, **2)** uses end-modified DNA strands and does not require an in-house DNA synthesizer, **3)** has short reaction time (about 2 hours), **4)** is easy to purify and **5)** importantly, it is highly modular, allowing the incorporation of different DNA sequences with controllable directionality, length and valency onto different synthetic molecules.

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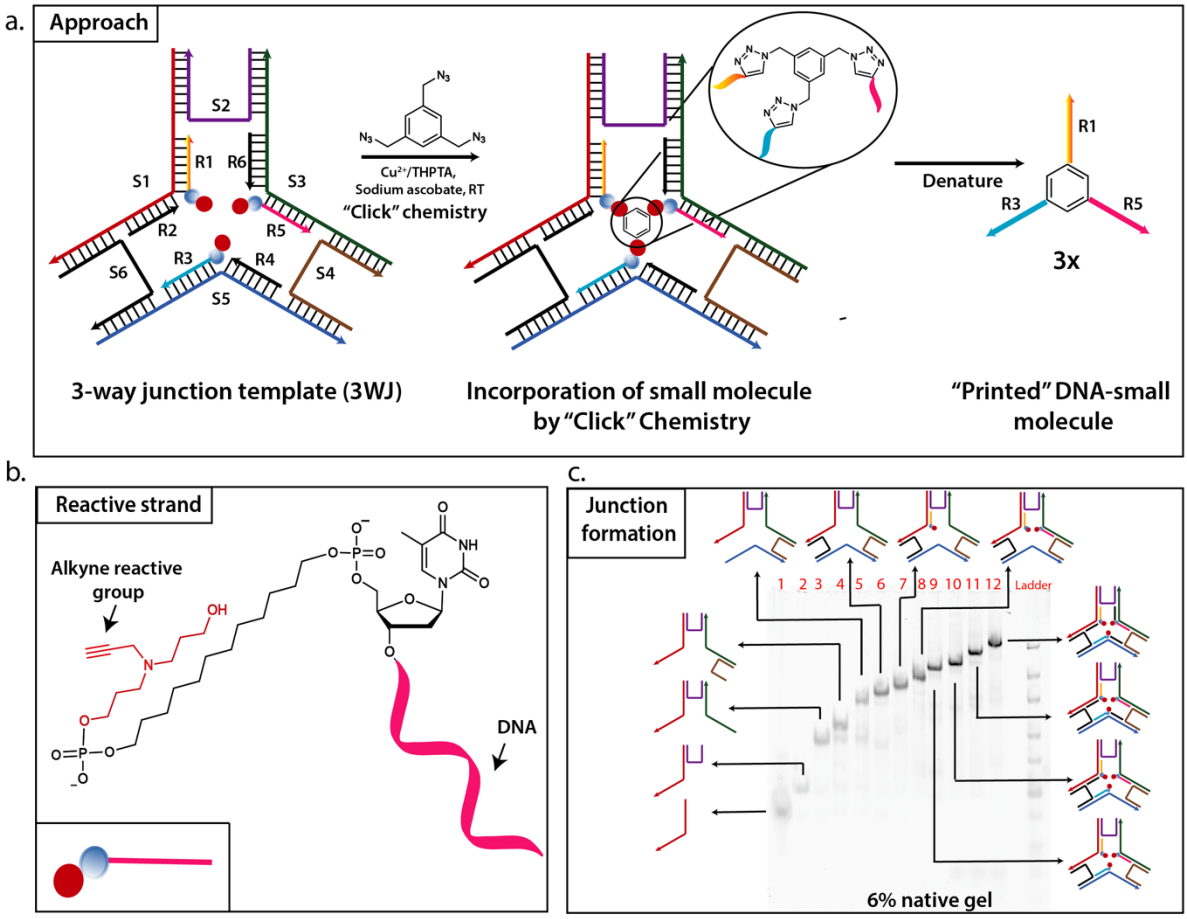

Figure 1. a) Schematic representation of the overall design approach, **b)** Chemical structure of reactive strand having 12-carbon organic spacer and alkyne group and **c)** 6% native PAGE shows stepwise assembly of the 3WJ.

Our strategy uses a self-assembled 3-way junction (3WJ) as a template (Fig. **1a**) containing six scaffold strands **S1** to **S6** in addition to single-stranded DNAs (ssDNAs) hybridized to the middle region of the junction (**R1** to **R6**). Unlike a typical DNA tile,[19] we break the long middle strand into six individual strands with three pointing their 5'-end and the other three pointing their 3'-end toward the middle region, in an alternating fashion. In this region, we hybridize two types of DNA strands: reactive and rigidifying strands. A reactive strand contains an alkyne functional group at its end and is separated from the DNA part by a commercially available hexaethylene (C12) spacer (Fig. **1b**), while the rigidifying strands without functional groups are hybridized to increase the junction's geometric definition. Once all the reactive and rigidifying strands are in pre-designated positions, this region would have high local concentration of the reactive groups and a small molecule would be able to react and "pick up" the alkyne-functionalized DNA strands covalently. The resulting DNA-small molecule product can be then released from the junction template by denaturation (Fig. **1a**). Figure **1c** shows a native polyacrylamide gel electrophoresis (PAGE) as the outcome of the stepwise 3WJ assembly. Lane **12** shows a near quantitative formation of the junction, which is robust enough to not fall apart at room temperature.

To examine our hypothesis, we first attempted to "print" three unique DNA strands (19 bases each) from a designed 3WJ to a 1,3,5-tris(azidomethyl)benzene core (Fig. **1a**). Previously, this core was used to connect to different DNA strands via solid-phase synthesis.[3c, 15d] However, instead of multi-step synthesis of the small molecule core, our method allows simple preparation of the core as well as the template from commercially available materials. The middle part is functionalized with 3 reactive 5'-alkyne-DNA strands, **R1, R3** and **R5**, and 3 rigidifying unmodified strands, **R2**, **R4** and **R6** (Fig. **1**). The denaturing gel in Fig. **2a** shows that the trimer with 3 different DNA sequences (**R1, R3** and **R5**) **(3x)** was formed with ~ 30% yield after 2 hours, along with rigidifying strands, scaffold strands **(S)** and side products (i.e. monomer and dimer). This represents an average yield of ~ 70% for each of three "click" reactions on the core. Note that there was low or no yield of trimer formation in the absence of template under the same conditions (Supporting Info-**SI-X**). To characterize the sequence asymmetry of **3x**, 3 fully complementary strands (**R1', R3'** and **R5'**) which hybridize with each of the arms of **3x** were added sequentially. Fig. **2b** reveals a gradual decrease in mobility of the structures, suggesting successful hybridization of the individual complementary strands (see **SI-VI** for mass spectrometry confirmation).

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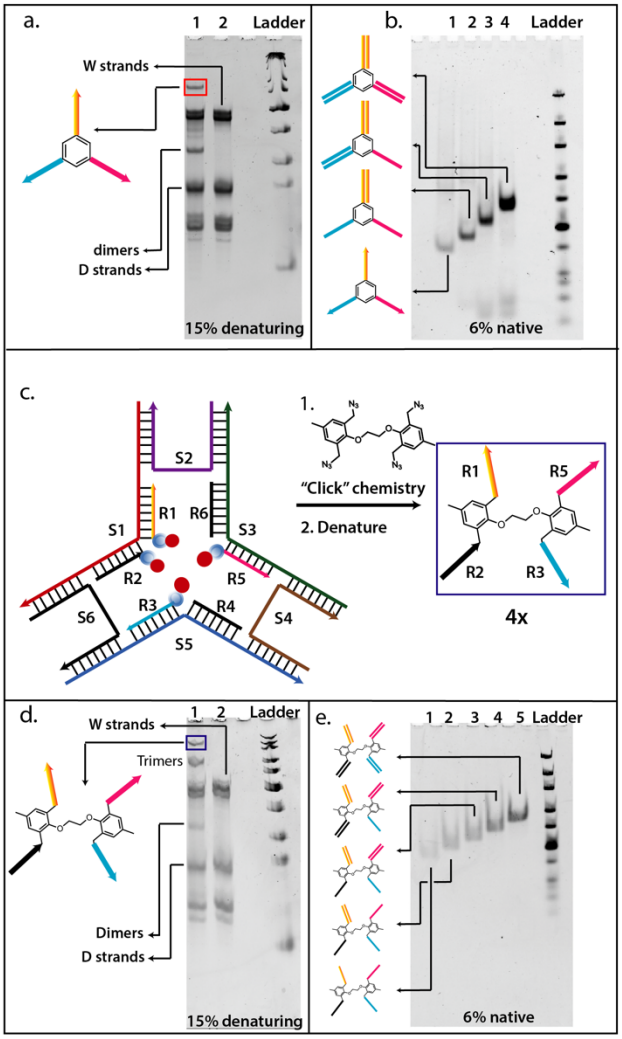

Figure 2. **a)** 15% denaturing PAGE for trimer formation. Lane 1: crude reaction mixture between fully assembled 3WJ with triazide molecules. Lane 2: fully assembled 3WJ in denaturing condition. **b)** Stepwise addition of complementary strands to **3x c)** Schematic representation and native **PAGE** of the addressability of the **3x**. **d)** Stepwise addition of complementary strands to **4x**.

We were interested in changing the directionality (5'-3') of the transferred DNA strands onto the core. The 3WJ allows us to easily switch the reactive alkynes to the 3' ends of the 3 other strands (**R2, R4** and **R6**), pointing toward the core of the 3WJ. We obtained a similar yield of the reverse direction trimer (with the 3'-ends of the DNA strands connected to the small molecule core) that would be difficult to achieve using previously reported methods **(SI-XI)**.[13-14,15d,16] Moreover, we were able to covalently link three DNA strands with different lengths (19-, 36- and 41-mer) to the benzene core **(SI-XII).**

To broaden the scope of this approach, we examined the transfer of 4 unique DNA strands onto a flexible tetraazide-functionalized molecule (Fig. **2c**). We hybridized 4 reactive strands (3 of them with 5'-alkynes and 1 with 3'-alkyne) and 2 rigidifying strands to the 3WJ (Fig. **2c**). The denaturing gel in Fig. **2d** reveals the formation of the tetramer product **(4x)** with a yield of 26% (~70% yield for each reaction). Similarly, the sequential decrease in mobility when hybridizing to complementary strands is shown in the native gel in Fig**. 2e**, confirming that **4x** indeed has 4 different strands grafted on the core. This approach thus offers full control over the number, sequences, and directionalities of the DNA strands transferred to the small molecule core.

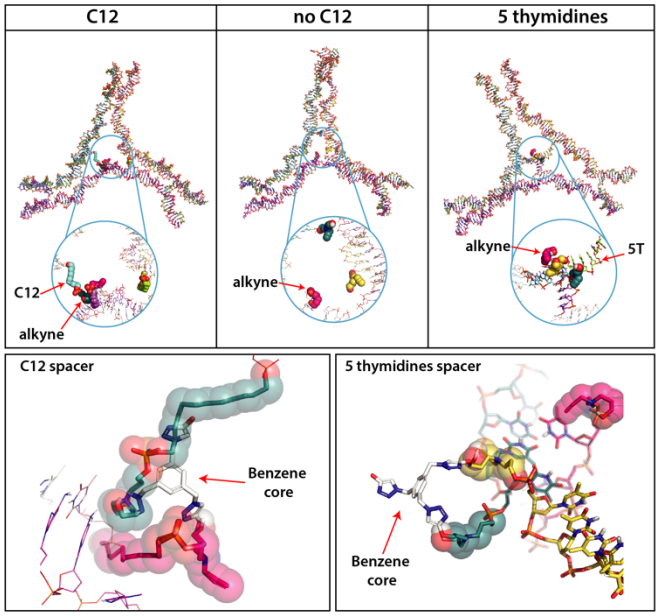

Figure 3. 3WJ models from MD simulations with 3 different reactive strands in the center with zoom-in top/side views on the right.

We were then interested in studying the effect of linker length, connecting the DNA portion to the alkyne moiety, on the efficacy of the transfer process. Interestingly, we observed that the trimer formation efficiency significantly decreased to 7% and 17%, by shortening the 12-carbon linker to 3-carbon and 6-carbon, respectively. When we completely removed the organic spacer and connected the DNA strand directly to the alkyne monomer, there was no trimer formation (see **SI-XIV).** We hypothesized that the length of the spacer plays a critical role in bringing the alkyne groups near the azides on the small molecule. Molecular dynamics (MD) simulations of the 3WJ (Fig**. 3**) were performed to gain insight into the molecular level. To understand the regulation of the reactive strands in the junction to the final small-molecule products, we simulated the 3WJ with three reactive-strand models: **1)** the C12 spacer (~18 Å) connecting the DNA and the alkyne; **2)** without the C12 linker; **3)** a longer spacer of 5 thymine bases (~24.5 Å). Simulations show that the spacer length indeed affects the inter-reactive strand distance towards the product yield. Within 30 ns, there is at least one pair of reactive strands within ~15 Å measured by nitrogen-nitrogen distance (in alkyne modifier) in the C12 spacer system **(SI-XVI).** In contrast, in the no-C12 spacer system, no reactive strands were found to maintain within 15 Å. When we replaced the C12 spacer with a longer 5 thymidine spacer, three reactive strands were able to reside closely together within ~15 Å. Thus, we predicted a spacer with length ~18 Å is essential to yield desired products. In some cases, having a long organic linker is not highly desirable (e.g. in terms of flexibility and hydrophobicity). Based on our simulations, we

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experimentally examined trimer formation by directly linking alkyne-functionalized DNA (no organic spacer) to the organic core. We substituted the C12 spacer with a 5-thymidine spacer and we observed formation of the trimer with ~35% yield, which is slightly improved compared to that using 12-carbon linker **(SI-XIV).** These results demonstrate that the spacer length is crucial for the process, and that direct linking of DNA to the small molecule core is efficient.

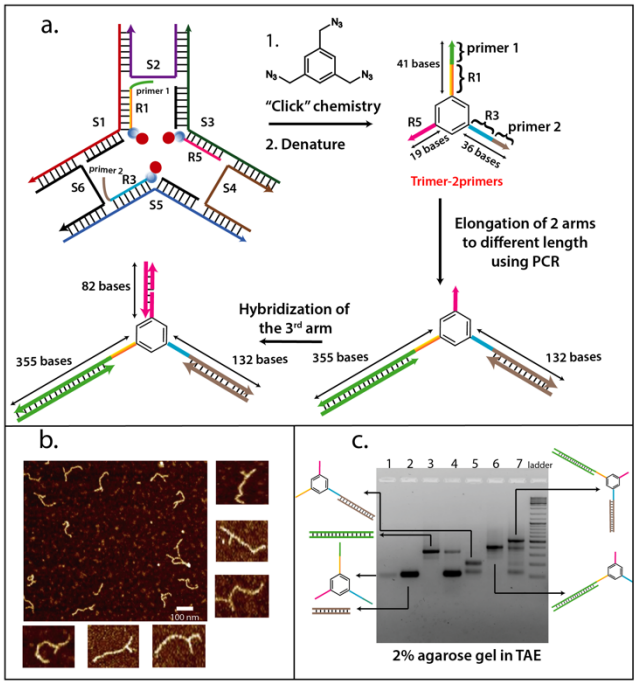

Figure 4. a) Scheme for DNA arm elongation using PCR. **b)** AFM images (in dry condition) confirm that the elongated trimer structure has exactly 3 arms of different lengths. **c)** 2% agarose gel in TAE buffer confirms the elongation of the DNA trimer structure. **Lane 1:** trimer-2primers (control); **Lane 2:** PCR product of ssDNA (113 bases)**; Lane 3**: PCR product of ssDNA (336 bases); **Lane 4:** PCR products of both ssDNA (113 bases) and ssDNA (336 bases) in the same mixture; **Lane 5:** Elongation of 1 arm in trimer to 132 bases in length; **Lane 6:** Elongation of 1 arm in trimer to 355 bases in length; **Lane 7**: Elongation of 2 arms simultaneously in trimer.

Long strands of DNA with controlled sequences are of interest for many applications including data storage, material organization and molecular electronics.[20] However, only up to 200 bases ssDNA can be practically obtained using conventional solid-phase synthesis. Therefore, simply hybridizing the 3-arm template with a long DNA strand will be limited by the length of ssDNAs made by a DNA synthesizer. Moreover, the stability of the construct would be highly dependent on a short hybridization region with the 3-arm template. One reliable method to elongate a DNA strand is PCR. Previously, Bao *et al.* demonstrated extension of identical DNA arms to micron-sized structures from small molecule cores, but the asymmetrical elongation of DNA arms is still a challenge.[21] Asymmetric DNA elongation can significantly enhance the complexity and information content of a DNA building block, that can potentially serve as a unique scaffold for DNA metalation and for organizing precisely different materials (e.g. quantum dots and/or gold nanoparticles). To probe the utility of our method, we set out to elongate 2 arms (**R1** and **R3)** of the asymmetric trimer simultaneously to different lengths using PCR. Since our method enables full control over sequence and length of the DNA strands grafted on the small molecule, we were able to synthesize a benzene core (called **trimer-2primers** – Fig. **4a**) connected to 3 different DNA arms: a 41-nucleotide (nt) arm containing a forward primer 1 (**R1-primer1**), a 36-nt arm containing a forward primer 2 (**R3-primer2**) and a 19-nt arm (**R5**). Two different long double-stranded DNA (dsDNA) were used as templates. One of the sequences was generated using 'temporal growth', a process previously developed by our lab, while the other was made using standard phosphoramidite DNA synthesis.[20a] Lane **7** in the agarose gel (Fig. **4c**) revealed the formation of the PCR desired product with a yield of 70-80% (3 repeats). Then, the band of interest was excised and further characterized by atomic force microscopy (AFM) under dry conditions. For easier visualization, we hybridized the third arm with an 82 bases dsDNA. Unambiguously, AFM confirmed that the product has exactly 3 arms, each with a different length (Fig. **4b**). The longest arm was measured to be 119 ± 9 nm (N=100), which is in accordance with the expected length of 121 nm. The two other arms were 46 ± 5 nm (N=100, theoretical length for 132 bp dsDNA ~ 45 nm) and 28 ± 4 nm (N=100, theoretical length for 85 bp dsDNA ~ 29 nm), respectively (see **SI-XV** for gel characterization). This is the first time a branched DNA-small molecule motif has been elongated asymmetrically using PCR.

DNA is one of the most classical examples of self-replication - the ability to make its own copies in nature. This concept has been recently transferred from living cells to DNA nanotechnology to replicate DNA tiles and DNA origami rafts.[22] Working towards this path may solve the problem of DNA structure scalability. Therefore, we would like to see if our asymmetric branched trimer structure (**trimer-2primers** in Fig**. 4**) can serve as a mother template to produce daughter generations chemically. Previously, von Kiedrowski *et al*. introduced this concept using a symmetrical junction.[23] First, we hybridized alkyne-functionalized **T2-R1comp, T3-R3comp** and **T4-R5comp** to the 'mother' template (**trimer-2primers**), followed by the addition of the triazide molecule. After denaturation, the 'daughter' structure, characterized by a higher gel mobility shift, was formed (Fig. **5b**-lane **1**), and the biotinylated 'mother' template was recovered using streptavidin-functionalized magnetic beads. The product appeared as a tight band by gel electrophoresis, suggesting that a single trimer was formed with a yield exceeding 90% (Fig. **5b** lane 3). Control experiments without template under the same conditions showed no branched trimer formation (Fig. **5b** – lane **2**). In addition, after 3 rounds of replication (Fig. **5a**), the daughter was amplified 3 times more than in a single cycle **(SI-XVII).** Our future work will focus on the exponential growth of both 'mother' and 'daughter' structures.

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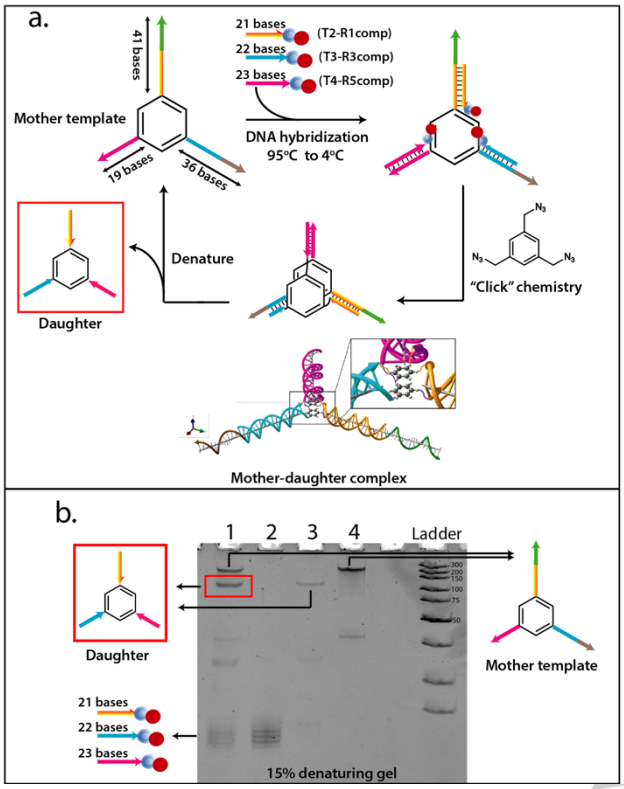

Figure 5. a) Making a copy from mother template (**trimer-2primers**) **b)** denaturing PAGE monitors the process. **Lane 1**: crude reaction mixture containing mother template, daughter, excess complementary ssDNA. **Lane 2:** control sample prepared under the same reaction condition without the mother template. **Lane 3:** reaction mixture after removing mother template and excess ssDNA complementary using streptavidin- magnetic bead. **Lane 4:** mother template **(trimer-2primers)** (control).

In summary, we have demonstrated a facile method to "print" different DNA strands onto a small molecule core with controllable valency, DNA directionalities and sequences. While we initially used a C12 spacer, MD simulations and experiments showed that DNA strands can be directly attached to the core. The DNA-imprinted small molecule can be extended asymmetrically using PCR and it can be chemically self-replicated to make a daughter generation. Our next step aims to control the "printing" process using external stimuli such as pH, strand displacement or other added biomolecules, an approach that can yield sensitive biological detection tools. The ease with which multivalent DNA-small molecule hybrids can be synthesized and purified will make them useful in the field of DNA nanotechnology as building blocks for wireframe DNA nanoobjects, branching staple strands in DNA origami and tunable templates for logic gates and material organization. By blending together DNA with synthetic molecules, DNA nanotechnology can acquire new structural motifs and can impart functionality to its typically passive DNA structures.

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Keywords: DNA nanotechnology • DNA printing • DNA-templated reactions• Self-assembly • Branched DNA structures

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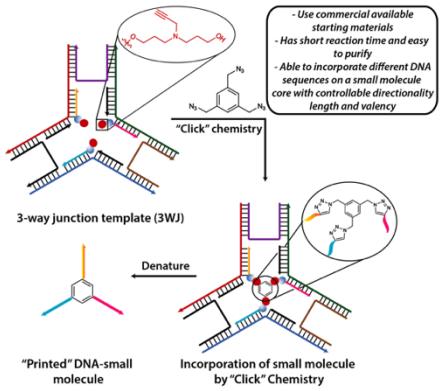
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Breaking the symmetry of small molecules using DNAs: A highly modular method to covalently transfer different DNA sequences with controllable directionality, length and valency onto different synthetic molecules is reported. These DNA-imprinted small molecules can be elongated asymmetrically to different length in high yield using polymerase chain reaction and can be chemically replicated.



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"Printing" DNA Strand Patterns on Small Molecules with Control of Valency, Directionality and Sequence