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Evolution of Structural DNA Nanotechnology

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The research field entitled structural DNA nanotechnology saw the light of day in the beginning of 1980s as the first immobile synthetic nucleic acid junctions were postulated and demonstrated. Since then, the field has taken huge leaps towards advanced applications, especially during the past decade. In this progress report we summarize how the controllable, custom and accurate nanostructures have recently evolved together with the powerful design and simulation software, and thus simultaneously provided a significant expansion of the shape space of the nanostructures. Today, researchers can select the most suitable fabrication methods, design paradigms and software from a variety of options when creating unique DNA nano-objects and -shapes for a plethora of implementations in materials science, optics, plasmonics, molecular patterning and nanomedicine.

1. Brief History: from Holliday Junction to Programmable and Periodic Structures

1.1. Motivation

The main purpose of DNA nanotechnology is to create artificial rationally designed nanostructures for diverse applications in biology, chemistry and physics.[1],[2] DNA is well-known for its essential role in all living systems as it carries genetic information. However, in the field of nanotechnology, DNA molecules can be utilized as a high-capacity information
storage, and interestingly, DNA molecules can be used as construction material to assemble accurate, biocompatible and functional nanostructures.

The founding father of structural DNA nanotechnology, Nadrian ‘Ned’ Seeman, realized in the beginning of 1980s that DNA molecules could be used as building material in creating predesigned DNA junctions for controlled bottom-up nanofabrication. The idea is based on the programmable and unique feature of hybridizing sequence-complementary parts of single-stranded DNA (ssDNA) molecules into double-stranded DNA (dsDNA) domains via Watson-Crick base pairing. These structural building blocks / units can be further assembled into larger programmed shapes. A great deal of the first compelling DNA assemblies were based on tile-like structures (Section 1.2) that enabled fabrication of two- (2D) and three-dimensional (3D) crystals, but nevertheless, the huge upturn in the field was arguably the invention of ‘DNA origami’ (Section 2) technique.

At present, an ever-increasing number of research groups are exploiting programmable self-assembly properties of nucleic acids in creating rationally designed nanoshapes and nanomachines for many different uses. In this progress report the main focus is on the different design paradigms (DNA origami and the related techniques are particularly emphasized), selected high-quality shapes and the software (Section 3) that enable user-friendly design and fabrication of DNA nano-objects.

1.2. First Structures and the Increasing Complexity

The very first DNA nanostructures were introduced by Ned Seeman as he had the idea that other molecules such as proteins could be arranged into well-ordered lattices for crystallography with the help of DNA-based scaffolds. These structures were based on a Holliday junction-like constructs, where ssDNA strands were linked together to form immobile junctions (Figure 1a, left). Through the predesigned ssDNA overhangs – so-called sticky-ends – at each arm, these junctions could be stitched together to form larger assemblies.
(Figure 1a, right). Soon after, these branched and immobile junctions were generalized allowing design also in three dimensions (3D). In 1991, the very first 3D DNA nano-object, a DNA cube was demonstrated (Figure 1b).\textsuperscript{[14]} In this design, the branch points of the junctions (or motifs) serve as vertices, and the strands (sticky-ends) from the branched molecules hybridize together thus forming rigid dsDNA domains, \textit{i.e.} the edges of the cube. After these discoveries, Fu and Seeman introduced a double-crossover (DX) molecule where two parallel DNA double helices are connected \textit{via} crossovers.\textsuperscript{[15]} In more detail, a DX tile can be created as shown in Figure 1c (Tile A): five unique strands (one green strand, two red and two blue ones) hybridize to each other and therefore they form two adjacent and immobile dsDNA motifs with two crossover junctions between them (compare to a single Holliday junction that is free to rotate). The DX motif was employed for forming robust periodic structures with the help of a sticky-end approach, and this strategy served as the basis for the first well-defined 2D arrays reported by Winfree \textit{et al.}\textsuperscript{[16]} (see Figure 1c for the DX tile motif and the principle DX tile –based 2D lattices). Later on the double-crossover design led to the development of a triple-crossover (TX) molecule consisting of three parallel dsDNA motifs held together (in a plane) \textit{via} two crossovers between each double helix.\textsuperscript{[17]} TX-tiles found more versatile uses, and besides diverse lattice geometries, \textit{e.g.} DNA nanotubes were formed using TX motifs as the building blocks (Figure 1d).\textsuperscript{[18],[19]}

Overall, as the researchers got inspired with these paradigms in the early 2000s, there started to be more and more design methods and their follow-up extensions that intensified the progress in the structural DNA nanotechnology. Examples based on these design principles include diverse polyhedral,\textsuperscript{[20]} 2D (and 3D) lattices with different types of branched junctions,\textsuperscript{[9],[21-23]} nanowires\textsuperscript{[24]} and various programmable tubular shapes.\textsuperscript{[25-28]} However, many of these techniques were quite sensitive to stoichiometric ratios of strands and the final object-size is challenging to control. Partially for this reason, some of these methods have been replaced with a highly versatile and in many cases more robust DNA origami
method. Nevertheless, (near) quantitative yields of pre-designed molecules and structures can be achieved via careful optimization. It is noteworthy to mention that these non-origami design paradigms provide an alternative access to simple DNA-minimal structures that can enable high level of complexity for many practical applications. Importantly, these abovementioned design paradigms have been successfully used in creating large 3D crystals – the long standing goal for DNA nanotechnology – based on 3D tensegrity triangles (Figure 1e). Tensegrity triangles are robust 3D DNA motifs consisting of three dsDNA domains – they are not in the same plane but have alternating over-and-under structure – and a three-fold rotational symmetry. The dsDNA domains of one motif are connected to other triangles via sticky-ends pointing to six different directions (from the ends of the helices). Due to the rotational symmetry of the design and the fact that six sticky ends form three identical complementary pairs, the adhesion of these triangles produces periodic 3D rhombohedral crystals (three independent directions of the crystal are shown in red, green and yellow in Figure 1e). Moreover, the diverse and truly programmable tiles can be used in algorithmic self-assembly (Figure 1f) thus enabling DNA-based computing, neural networks, kinetic growth and fully dynamic DNA nanotechnology (can be achieved by strand displacement reactions).
Figure 1. Designs based on immobile junctions, tiles and sticky-ends. A) The original idea of using four-ssDNAs to form immobile branched designs that can be combined into a network. Reproduced with permission.\textsuperscript{[36]} 2003, Nature Publishing Group. B) A cube made from DNA.\textsuperscript{[14]} Reproduced with permission.\textsuperscript{[36]} 2003, Nature Publishing Group. C) Double-crossover tile that can be programmed to form large assemblies / lattices.\textsuperscript{[16]} Reproduced with permission.\textsuperscript{[36]} 2003, Nature Publishing Group. D) Extension to double-crossover molecule is a triple-crossover tile that can be used to form e.g. lattices and DNA nanotubes. Reproduced with permission.\textsuperscript{[19]} 2004, National Academy of Sciences. E) Tensegrity triangles can be used to form large 3D lattices and DNA crystals. Reproduced with permission.\textsuperscript{[31]} 2009, Nature Publishing Group. F) Algorithmic pre-programmed self-assembly allows DNA computing and formation of e.g. Sierpinski triangles. Reproduced with permission.\textsuperscript{[33]} 2008, American Chemical Society.

2. DNA Origami and Beyond

2.1. The Birth of DNA Origami and the Source of Scaffold

In the beginning of this millennium, the field of structural DNA nanotechnology started to get more and more attention\textsuperscript{[36]} and simultaneously, the design techniques started to develop rapidly. The work by Shih \textit{et al.} in 2004 paved the way to rigid and well-defined DNA designs that can resist deformation.\textsuperscript{[37]} A so-called “pre-origami”, a nanoscale DNA octahedron was folded from a mixture of 1,669-nt scaffold and five 40-nt staple strands. The
octahedron comprised five double-crossover (DX) struts and seven paranemic-crossover (PX) struts, connected via six four-way junctions at the vertexes (Figure 2a), all joined together by heat denaturation and subsequent cooling steps.

Shih’s work truly inspired Paul Rothemund from Caltech, and in 2006 Rothemund’s transformational paper induced a big boom in the structural DNA nanotechnology, simultaneously starting an era of DNA origami architectures.\(^{[38]}\) Rothemund’s origami approach was based on folding a 7-kilobase single-stranded DNA scaffold strand into a desired and fully predefined 2D shapes, ca. 100 nm in size, with the aid of a set of short 20-60 bp oligonucleotides i.e. “staple strands” that lock the scaffold in place. Six different geometric models e.g. square, rectangle, star, disc with holes (smiley face, Figure 2b) and two types of triangles were designed and build using extracted circular genomic DNA from the M13mp18 bacteriophage. Since then, M13mp18 and its slightly extended versions (short inserts in the genome) have been the most frequently used scaffold strand options with a proven track record.

In practice, the size and the level of complexity of a DNA origami structure are limited by the length of the scaffold.\(^{[39]}\) However, a single-stranded DNA can be produced at various lengths using several strategies reported in the literature.\(^{[40]}\) For example, Zhang et al.\(^{[41]}\) used long-range polymerase chain reaction (PCR) for the amplification of a 26 kilobase fragment of λ-DNA (48,502 nt) in the double-stranded DNA form (dsDNA) followed by a selective enzymatic digestion to obtain long ssDNA scaffold. Marchi et al.\(^{[42]}\) developed a biological production method for 51,466 nt ssDNA derived from λ/M13 hybrid phage by controlling its mode of replication by propagation on one of two host bacteria. Recently, Kick et al. produced ssDNA in gram-scale per liter-reaction-volume by using high-cell-density fermentation of bacteriophage-infected E. coli in a stirred-tank bioreactor instead of shake flasks, a remarkable increase of two orders of magnitude in the efficacy of DNA scaffold production.\(^{[43]}\)
Long scaffold strands have also drawbacks, such as the strand cleavage and the need of large number of staple strands that makes the approach costly. Therefore, Said et al. prepared a smaller strand named as M1.3, obtained from M13mp18 using cleavage-inducing oligonucleotides and restriction endonucleases. The scaffold exists in a linear or cyclic form having 704 nucleotides which require only 15-24 staple strands for the assembly of small origamis like 2D triangle, curved six-helix bundle or 3D cube.[44]

In general, methods for creating long double-stranded DNA scaffolds (dsDNA) are considered more powerful than methods for ssDNA production. However, the use of dsDNA as such for the origami folding is challenging due to the enthalpy change needed to dissociate two complementary scaffold strands - this is far higher than the enthalpy change gained for binding the staple strands to the scaffolds. Högberg et al.[45] managed to bypass this obstacle by using a combination of fast temperature drop and gradual removal of added chemical denaturing agents in the presence of two distinct sets of staple strands to produce six-helix bundles and six-helix bundle triangles in a single folding event. Yang et al.[46] elaborated the dsDNA scaffold folding obtained from λ DNA by emphasizing two principles: firstly, folding path asymmetry to minimize unwanted complementarities between staple strands and secondly, periodic convergence of the scaffold strands.

2.2. DNA Origami Goes 3D

The translation from 2D origami structures to 3D objects was initiated by Andersen et al.,[47] Ke et al.,[48] and Douglas et al.[49] in 2009. Andersen and co-workers created the famous DNA box (42 nm × 36 nm × 36 nm) assembled from six 2D origami sheets (Figure 2c, left). The box contained a controllable lid functionalized with a dual lock-key system comprised of dsDNAs with single-stranded extensions. The attachment of two fluorophores, to the adjacent faces of the box enabled the detection of the lid opening by fluorescence resonance energy transfer (FRET). Reversible opening and closing of a lid in a hollow DNA origami box
having dimensions of 18 nm × 18 nm × 24 nm was demonstrated by Zadegan et al. in 2012. Ke and co-workers, for one, developed a DNA tetrahedron molecular cage (Figure 2c, right) having an estimated volume of $1.5 \times 10^{-23}$ m$^3$. In this particular design, the single DNA scaffold ran through the entire structure thus, forming four adjacent planar triangular faces, which were subsequently annealed with the staple strands to form a seamless DNA tetrahedron.

As a useful extension to 3D origami designs, Douglas et al. developed a method to create multi-layered 3D origami that is relying on the designer lattice. They created a computer-aided design platform caDNAno (see software section), which enabled simplified and rational design and manufacture process of generally more complicated DNA origami shapes. As a result, they were able to introduce more variety to 3D structures by exploiting honeycomb-pleat based strategy which facilitated the design and construction of shapes like monolith, square nut, railed bridge, genie bottle, stacked and slotted crosses, all having dimensions in the 10-100 nm range. However, the assembly and mixing of scaffold strands derived from the single-stranded genome of M13 bacteriophage and oligonucleotide staple strands required carefully calibrated monovalent and divalent cation concentrations and folding time of roughly one week. As a follow-up of this work, Ke et al. introduced even more compact 3D origami lattice where densely packed layers of helices were adopting square-lattice geometry. This can be considered as more beneficial form of architecture than the honeycomb lattice if flat surfaces are to be designed (compare to Rothemund’s original 2D designs). Subsequent work by Ke et al. extended the method by enabling close-packed multi-layered hexagonal lattice geometry as well as new hybrid lattice types, i.e. combinations of closed-packed, honeycomb and square lattices in one design, thus creating even more complicated hybrid DNA origami constructs. Besides just allowing more degrees of freedom to build in independent directions, 3D multi-layer structures provide also more robustness compared to single-layer 2D or 3D origami designs. As the single-layer structures tend to fluctuate and
deform significantly in the aqueous solution, minimization of the structural fluctuations may be an important feature in many solution phase applications. However, most 2D origami structures can find intriguing uses if they are supported by a substrate.

The abovementioned square and honeycomb packing geometries (Figure 2d) can be found as default lattices in caDNAano and simulation software CanDo. Together they provide a user-friendly approach to design 3D structures by sculpting the target shape from the lattice and predicting the curvature and twist of the shape in aqueous solution (see the software section for more details).

Dietz et al. introduced multi-layered origami structures with controllable twists, bends and curvatures (Figure 2e, left) through programmable self-assembly of a model system: a 10-row, 6-helix-per-row (10-by-6) bundle composed of 60 tightly interconnected DNA double helices. Systematic insertions and deletions of base pairs of the helices forced the 10-by-6 bundles to create a global twisting of either handedness or controlled bending of the bundle. By a combination of multiple curved elements several new complex nanostructures, such as a concave and convex triangles, wireframe capsule, or 12-toothed gears were obtained.

Related to Dietz’s work, Han et al. presented a slightly different approach to introduce complex curvatures to the origami shapes. Their design principles for DNA origami enabled curved structures in 3D space such as sphere, ellipsoid and nanoflask (Figure 2e, right) having cylindrical neck and rounded bottom. First, a 2D concentric rings and squares with rounded outer corners were generated by creating tension between adjacent helices. Bending in-plane was achieved by setting the distance between crossovers in the outer rings greater than in the inner rings. Second, out-of-plane curvature was implemented by shifting the particular position and pattern of crossover points between adjacent double helices in B-form.

Another strategy for complex geometries is to take advantage of tensional integrity - a reliance on a balance between components that are either in pure compression or in pure tension for stability. So-called “tensegrity” structures may exhibit extremely high strength-to-
weight ratios and great resilience. Liedl *et al.* used this feature for fabricating pre-stressed, 3D tensegrity prism composed of three compression-resistant 13-helix bundles held in place by nine tensed ssDNA springs that acted as tension-bearing cables. Moreover, they created various 2D tensegrity kites using two 12-helix bundles tied together through four unpaired regions of a 8,634-nt scaffold strand, which then provided necessary tension and compression to the connecting struts.[56]

All of the abovementioned methods are based on folding a long ssDNA with the help of shorter ssDNA strands. However, instead using a DNA scaffold, Wei *et al.* created a highly modular method to form complex 2D shapes by using DNA tiles.[57] A single-stranded tile (STT) is a 42 bp DNA strand composed entirely of concatenated sticky ends and which folds into a rectangular shape due to its interaction with four neighboring SSTs during self-assembly. Each strand has a unique sequence and therefore hundreds of distinct tiles can be self-assembled into a large rectangle. This rectangle serves as a molecular canvas, where each tile corresponds to a 3 nm × 7 nm sized pixel. A desired shape, sketched on the canvas pixel by pixel, was then produced by annealing of all those STTs that corresponded to pixels covered by the target shape, e.g. eagle head (shown in dark blue in Figure 2f, left). The remaining strands (light blue color in Figure 2f, left) were excluded. Parallel to STTs Ke *et al.* created DNA-bricks which enabled the fabrication of complex prescribed three-dimensional shapes. Each DNA brick is a sequence-specific LEGO-like modular building block having voxel dimensions of 2.5 nm × 2.5 nm × 2.7 nm. More than hundred distinct shapes were constructed from 3D molecular canvas (10 × 10 × 10 voxel cuboid) *via* annealing specific subsets of bricks (such as the shape shown in Figure 2f, right).[58] As an extension to this work Wei *et al.* evaluated over 30 SST motifs that formed lattice structures with diverse strand weaving patterns and specific geometric properties. The systematic study provided new rules for the design space for complex DNA structures such as curvature, twist and corrugated sheets.[59]

2.3. DNA Origami from Meshes

Besides of the methods presented above, DNA designs can be prepared based on 3D meshing – a strategy well-known from macroscopic engineering – which is now applied to nanoscale fabrication. Han et al. were first to show a new meshing origami technique. Their flexible approach was based on a "gridiron unit" i.e. a series of four-arm junctions joined together to form a two-layer square frame. Designed distortion at the junctions enables a scaffold strand.
to traverse through individual vertices in multiple directions. A number of 2D- and 3D-structures having highly curved structures, such as a sphere (Figure 2g, left) or a screw were fabricated.\cite{60}

Benson et al.\cite{61} developed a general method and a software vHelix (see software section) of folding arbitrary polygonal digital meshes in DNA that provide access to complex 3D nanosized objects, ranging from an icosahedron (Figure 2g, middle) to a bottle, waving stickman and a Stanford bunny. The highly automated design process employed a routing algorithm based on A-trails, a specific type of Eulerian circuits, where consecutive edges of the circuit are always neighbours in the cyclic ordering around the vertices and a relaxation simulation that traces scaffold strands through the target structures. Moreover, the routing of staple strands that aid the DNA-origami folding follows implicitly from completing the edge connections at the vertices. As an extension, Benson et al.\cite{63} applied a closely related design concept to build flat sheet meshes. Matthies et al., for one, designed trianglated DNA trusses, \textit{i.e.} wireframe truss-structures composed of equilateral triangles.\cite{64} In general, these wireframe methods require less material per volume than standard multiple-helix bundles.

Arguably the most elaborate example of top-down wireframe design and of turning the design paradigm / fabrication method onto its head was demonstrated by Veneziano et al.\cite{62} (Figure 2g, right) who developed algorithms that fully automate the design of arbitrary DNA structures (DAEDALUS software, see software section). Library of 45 complex nanoparticle geometries including Platonic, Archimedean, Catalan, and Johnson solids, and over 10 arbitrarily shaped solids were designed. Each object were then represented as closed surfaces rendered as polyhedral networks of parallel DNA duplexes, which enabled to complete DNA scaffold routing with the aid of a so-called spanning-tree algorithm. Custom length ssDNA scaffolds were produced by asymmetric polymerase chain reaction (aPCR) to facilitate different sizes of the final object. High structural fidelity in 3D was verified by characterization with single particle cryo-electron microscopy.
2.4. Towards Larger Structures

Liu et al.\cite{65} prepared crystalline 2D origami arrays by using two distinct cross-shaped tiles, ca. 100 nm × 100 nm, of which helical axes were designed in two orthogonal directions (Figure 3a, left). Due to the multiple programmed sticky-ends at each connection site, the annealing temperature had to be optimized in order to form 2D DNA arrays up to few micrometers in size (Figure 3a, right). Zhao et al.\cite{66} presented four examples of “origami of origami” or “superorigami” by organizing DNA origami tiles into larger structures by using preformed scaffold frames (Figure 3b). Hexagon, square, hexagonal- and diamond-shaped superstructures with dimensions up to 220 nm × 375 nm were assembled via annealing. Besides these methods, surface-assisted hierarchical assemblies of DNA origami and tiles that yield large high-quality lattices have been demonstrated.\cite{67-70} These techniques can be realized on biological membranes and on MICA substrates, but they often need careful tuning of salt concentration for the optimal binding and assembly results. Other ways to create lattice-based systems are so-called inter-tile strategies \cite{71-73} and nanoparticle-mediated lattices, i.e. nanoparticle-DNA hybrid lattices.\cite{74,75}

As a different approach to grow larger structures, Iinuma et al.\cite{76} developed a concept for 3D wireframe polyhedrons self-assembled from DNA origami “tripod” monomers weighting ca. 5 MDa (Figure 3c). The versatile tripod features three equal-length stiff arms (50 nm length 16-helix bundles (16HB) connected at the vertex by two-helix bundle supporting “struts” with inter-arm angles of 60° or 90°. Tripods were joined together via “connector” strands having up to 30 base pairs. A series of polyhedrons were obtained in one-pot annealing process by first assembling tripod monomers which further self-assembled into the final shapes without the need of intermediate purification. As a result, a tetrahedron (~20 MD), a triangular prism (~30 MD), a cube (~40 MD), a pentagonal prism (~50 MD), and a hexagonal prism (~60 MD) with edges 100 nm in length were obtained. Sizes of these assemblies are comparable with
those of bacterial microcompartments such as carboxysomes, and therefore this approach extends the shape and size space thus providing the biologically highly relevant size range of rationally designed DNA nanostructures.

Ke et al.\textsuperscript{[77]} elaborated their previous DNA-brick design (32-nt strand)\textsuperscript{[58]} and introduced a robust concept to grow 2D DNA-brick crystals in their lateral dimensions up to micrometer size with precisely engineered depths up to 80 nm embedded with 3D pores, channels or tunnels with nanometer resolution (Figure 3d). Out of 32 brick crystals grown four types was distinguished: 1) Z-crystals: 1D ‘DNA-bundle’ crystals extending along the z-axis, 2) X-crystals: 1D crystals extending along the x-axis, 3) ZX-crystals: 2D ‘multilayer’ crystals extending along z- and x-axis, 4) XY-crystals: 2D ‘DNA-forest’ crystals extending along the x- and y-axis. Unlike the typical DNA crystal growth, which occurs \textit{via} a two-stage hierarchical process, the growth of DNA brick crystals in question was non-hierarchical, thus facilitating isothermal formation of crystals.

2.5. Alternative Methods for Building from the Bottom-Up

Gerling et al.\textsuperscript{[78]} added still another dimension for DNA origami preparation by adapting and emulating protein self-assembly which often occur \textit{via} shape-complementary interfaces that allow conformational dynamics due to weaker interactions than base pairing. Several precisely defined 3D DNA objects were designed and assembled in solution, which contained the protrusion and extrusion sites allowing dsDNA domains to be bound to each other \textit{via} blunt ends (Figure 3e). The conformation of the assemblies could be influenced by MgCl\textsubscript{2} concentration, solution temperature or allosteric regulation mechanism. Compilation of higher order filaments formed by cation-dependent reversible multimerization of a self-complementary multilayer DNA origami brick, hexagon or cross having lattices up a micrometer-scale was demonstrated. Furthermore, reversibly reconfigurable devices \textit{e.g.} an
actuator, a switchable gear, or a 15 MD heterotrimeric reconfigurable “nanorobot” that can switch shape into three different conformational states were elucidated.

As an interesting alternative to the methods presented above, Praetorius and Dietz developed a method where DNA molecules can be folded into desired shapes with the help of TAL proteins that are DNA-sequence specific (Figure 3f).[79] The authors studied in detail how TAL protein and the hybrid bridged TAL-proteins could bind to DNA, and therefore developed new designing rules for such structures. They demonstrated the feasibility of their novel method by introducing several shapes, such as structures with different curvatures and for example a square and a tile-like structure that resembles the original DNA DX tile. The absolute advantage here is that one does not need to anneal the structures, as the protein will find its target sequence and thus fold the DNA at room temperature. Moreover, all the components of these hybrid DNA-protein structures can be produced in vitro, and thus the method opens up an opportunity to produce the whole predesigned nanoscale shapes even in living cells. Besides TAL-proteins, there exist more possibilities to harness DNA-binding proteins for forming hybrid structures; Schiffels et al.[80] showed recently that also RecA proteins can be used for creating hybrid tetrahedral, rectangular and linear DNA-protein shapes.
3. Design Software and Simulation of the DNA Nanoshapes

3.1. The Earliest Design Software

The first design software for DNA nanostructures, GIDEON, was developed by Ned Seeman and colleagues in 2006, which was later on partially replaced with other software and techniques - however, GIDEON is still used in many labs. After the dawn of DNA design software, the researchers quickly adopted new and revolutionary DNA origami method, and subsequently, this effort yielded first origami-oriented software SARSE and soon after, even more advanced tools.
SARSE developed by Andersen et al.\cite{82} was the earliest publicly accessible tool for designing DNA origami structures. It contained some special features: bitmap reader, automatic “origami folding system”, 3D generator and oligotracker. According to the original article, the origami folder is able to automatically find a folding path through the shape and to add staple strands with crossovers as well. 3D generator yields an atomic model of the structure and finally, the oligotracker is used to insert a given sequence of nucleotides in the scaffold and export an output list of staple strands and their sequences.

3.2. caDNAno and CanDo

caDNAno by Douglas et al.\cite{83} and CanDo by Castro et al.\cite{53} and Kim et al.\cite{84} are widely used together for designing 2D and 3D DNA origami nanostructures and computationally predicting the actual shapes of the designed structures in aqueous solution.

The core purpose of caDNAno is to simplify and speed up the design process of DNA nanostructures. The usual design process in caDNAno is divided into four steps. First, the desired shape is approximated by inserting helices into the predefined lattice (either honeycomb or square lattice geometry). There is either a direct visualization tool (older versions) or a 3D interface provided by Autodesk Maya (newer versions) that guides the eye in the designing process. Next, the scaffold path that passes between neighboring helices is constructed (the scaffold path has to include antiparallel crossovers to connect neighboring helices). The goal is to construct a scaffold path in a rasterized manner meaning that the path is continuous from the beginning to the end. Once the scaffold routing is completed, staple strands are inserted. By default, caDNAno inserts the allowed staple crossovers, and then the staple strands can be split into shorter segments by hand or automatically. Finally, the desired DNA sequence is inserted into scaffold and the complementary staple strand sequences are generated.
The lattice grid is divided by 7 base pair segments for the honeycomb lattice and in 8 base pair segments for the square lattice geometries. The 8 base pair rule was used already in Rothemund's seminal work[38] where the 2D structures were essentially single-layer structures in square lattice geometry. In caDNAno one can tune the curvature, twist or bend of the structure by deleting or inserting base pairs of the design.[54] Deletion of base pairs from the segments leads to a left-handed torque and inserting additional base pairs induces a right-handed torque. This feature can be controlled to some extent, since the twisting or bending can be simulated by CanDo software.

Overall, caDNAno is intuitive enough to use, and important visual clues, such as marked crossover positions, make it user-friendly. Twists and bends are not directly supported, but as described above, it is possible to skip and insert base pairs at desired locations. Unfortunately, there is no direct visualization for this feature and for that reason designing such structures might be tedious. For such features, powerful simulation software is needed.

As already briefly mentioned, CanDo is a tool for predicting appearance, mechanical fluctuations and flexibility, as well as twists and bends of the particular shape in the solution. The software was developed by Hendrik Dietz's (Technische Universität München) and Mark Bathe's groups (Massachusetts Institute of Technology) and it is currently maintained by the Laboratory for Computational Biology and Biophysics at MIT. The idea is to offer computational feedback (finite-element based rigid-beam model) for reducing financial costs and time to design nanostructures successfully (try-and-error process). For example, it can be used to verify wrongly designed or otherwise undesired structural features already before synthesis. CanDo directly supports caDNAno project files (.json), and is therefore often used in conjunction with the caDNAno design procedure. CanDo can be efficiently used to approximate the shape of the origami structure before the synthesis for many practical applications. For instance, one can simulate how bent or twisted the structure is, and try to iterate the design parameters in order to get a desired result. Importantly, if the resolution of
CanDo simulation is not enough for practical uses, one can also use fully atomistic molecular dynamics simulations to validate the structure of DNA origami.\[^{85}\]

3.3 Tiamat

Tiamat software\[^{86}\] was developed in 2009 by the researchers of Hao Yan’s Lab at the Arizona State University. It was developed to overcome some limitations of GIDEON (and UNIQUIMER 3D\[^{87}\]) software, and it has specifically been designed for more complex DNA nanostructures.

The most important feature in Tiamat is that it uses lattice-free approach as opposed to caDNAno. Recently introduced wireframe technique DAEDALUS\[^{62}\] (see section 3.5) and its lattice-free simulation\[^{88}\]/visualization tool are partially based on the Tiamat software features (same simulation tool can be used to simulate Tiamat design files (.dna)). Tiamat is based on combining double-stranded DNA elements together by hand (visually), and therefore it can be easily used to create geometries, such as different tiles or wire-frame-like objects (for example a DX tile, which currently serves as an edge of all DAEDALUS shapes) or a tetrahedron. However, at present, there are other versatile lattice-free techniques available.

3.4. vHelix

vHelix software developed by Björn Högberg’s group at the Karolinska Institutet\[^{61}\] aims to overcome the limitations of previous design software, such as the complexity of the overall design process. The core idea is to automate the scaffolding process and thus eliminate extra manual work by hand, and therefore allow a user to create more elaborate structures. vHelix uses routing algorithm based on graph theory and a relaxation simulation that creates scaffold strands using a given target structure (3D model) as an input. vHelix works for structures that enclose a volume, which can be inflated into a ball (spherical topologies) and the fundamental idea is to replace all the edges of the target mesh with single DNA double helices.
Furthermore, the constructed scaffold strand should only traverse each of the edges once, thus forming the Chinese postman problem: there is a closed ‘walk’ covering every edge only once. In addition, there are three principles that need to be satisfied. First, meshes should be triangulated in order to increase structural rigidity. Second, each edge of the target mesh should be represented by just a single double helix to keep the amount of used DNA as low as possible hereby enabling construction of large structures, and finally, the scaffold should not cross itself at the vertices minimizing topological and kinetic traps during the folding procedure. vHelix runs as a plugin in Autodesk Maya, and the scaffold routing algorithm with the spring relaxation can be found from a separate software called BSCOR.

As Tiamat (see above), vHelix is also lattice-free software, and the structures constructed with vHelix are based on the wireframe approach. The first step in the workflow is to construct a polyhedral 3D mesh of the target nanoscale geometry using 3D modeling software. Next, the long scaffold strand that traverses through all the edges of the mesh, is generated. The least-strained DNA helix arrangement is determined and finally the generated staple strands can be further tuned by a user. For example, staple-strand breakpoints can be modified. After these adjustments vHelix automatically generates the staple-strand sequences. The final structure is comprised of the maximum number of single duplex edges (maximized by the scaffold routing algorithm) and therefore it minimizes the amount of DNA used in the design. Recently, the developers of vHelix introduced an extension to the original software. This extension allows one to create 2D origamis using flat-sheet meshes.\(^{63}\)

3.5. DAEDALUS

The DAEDALUS\(^{62}\) (DNA Origami Sequence Design Algorithm for User-defined Structures) project is maintained by the Laboratory for Computational Biology and Biophysics at MIT by Mark Bathe and colleagues. The ultimate goal of DAEDALUS is to allow anyone (including non-experts of DNA nanotechnology) to design and synthesize their own DNA assemblies.
DAEDALUS is fully automated and supports almost any target 3D geometry. Only an input CAD file is required, from which DAEDALUS will generate the necessary DNA sequences that are needed for creating scaffolded DNA origami (as explained earlier).

DAEDALUS is another wireframe approach, where the DNA sequences follow the edges of the 3D object. The differences between vHelix and DAEDALUS are that in the latter human input is reduced to a minimum and it also supports greater amount of different sizes and topologies. In addition, DAEDALUS is based on the more rigid double-crossover (DX) edges, whereas vHelix only supports single duplex edges.

As the starting point in the workflow, the graph of the target structure is constructed by using the given target mesh, and it includes vertex, edge and face information. Next, a spanning-tree algorithm is used to generate a route for the scaffold strand to run through the target shape while ensuring that the scaffold does not intersect itself at vertices. After the scaffold routing is determined, staple strands are generated automatically, and finally a 3D atomic model is generated to predict the final structure. It is noteworthy that there exist two ways to use DAEDALUS: using the online version or running the software locally on MATLAB.

3.6. Summary of the Design Software

The main features of the above-discussed software in are summarized, commented and listed in the Table 1.

<table>
<thead>
<tr>
<th>Functionality / feature</th>
<th>caDNAno</th>
<th>Tiamat</th>
<th>vHelix</th>
<th>DAEDALUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottom-up / Top-down</td>
<td>Bottom-up</td>
<td>Bottom-up</td>
<td>Top-down</td>
<td>Top-down</td>
</tr>
<tr>
<td>Shape Space</td>
<td>2D/3D</td>
<td>2D/3D</td>
<td>2D/3D</td>
<td>2D/3D</td>
</tr>
<tr>
<td>Lattice type</td>
<td>Lattice (honeycomb or square)</td>
<td>Lattice-free</td>
<td>Lattice-free</td>
<td>Lattice-free</td>
</tr>
<tr>
<td>Wire-frame</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Visualization</td>
<td>Maya / CanDo</td>
<td>Direct visualization / CanDo</td>
<td>Direct visualization with Maya</td>
<td>Direct visualization, atomic model / CanDo</td>
</tr>
<tr>
<td>Automatization (scaffold routing)</td>
<td>No</td>
<td>No a)</td>
<td>Yes b)</td>
<td>Yes c)</td>
</tr>
</tbody>
</table>
Automatization (staple design)

<table>
<thead>
<tr>
<th>Input</th>
<th>Automatization (staple design)</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>.json (read)</td>
<td>.json (read)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>.dna (read)</td>
<td>.poly (routing from BSCOR)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>.poly (models), .mat (previously generated routing)</td>
<td>.poly (models), .mat (previously generated routing)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Output (design / staples)

| .json (for CanDo), .csv (staples) | .json (for CanDo), .csv (staples) | .csv (staples) | .csv (staples) |
| .dna (for CanDo), .avi (cartoons), .txt (staples) | .dna (for CanDo), .avi (cartoons), .txt (staples) | .csv (staples) | .csv (staples) |
| .csv (staples), .cnodo (for CanDo), .tif, .pdb, .bild (atomic models) | .csv (staples), .cnodo (for CanDo), .tif, .pdb, .bild (atomic models) | .csv (staples) | .csv (staples) |

a) Structures are created by combining dsDNA motifs together. However, the software generates a list of staple sequences as an output.
b) Mesh size can be tuned by scaling factor before scaffold routing.
c) Mesh size (edge length) can be modified in MATLAB code.
d) Creates staples automatically, but the strands have to be cut manually or by using the autobreak option. However, staples can be modified manually in an intuitive way.
e) Staples can be modified by hand and they are visualized in Maya.
f) Currently it is challenging to modify individual staples by hand once the structure is created.

Note 1: vHelix currently supports only single duplex edges and spherical topologies, whereas DAEDALUS enables more rigid double-crossover edges.
Note 2: All the software accept customized scaffold lengths and sequences, but only the original DAEDALUS article provides methods to produce (linear) custom-length scaffold strands.

4. Applications and Future Perspectives

Despite the rapid progress in designing versatile structures and in developing fabrication methods reported here, many implementations of DNA nanotechnology are still quite impractical. However, there exist already implementations for helping researchers to map the atomic structure of proteins, computing in living cells, and soon, prospectively, tracking and curing diseases with DNA structures might become possible. In this section we summarize the latest advances in the design optimization and list some recent and notable examples how DNA nanotechnology can be harnessed in a number of subfields of materials science, biotechnology and medicine.

4.1. Design Optimization

As an important step for getting closer to practical applications, a lot of effort has been put into optimization of designing paradigms and folding conditions of DNA nanostructures. It
has been shown the structures can be rapidly folded at constant temperatures\textsuperscript{[89],[90]} and that there are ways to increase the folding yields\textsuperscript{[91],[92]} while avoiding the possible kinetic traps in the folding procedure. For many implementations, it is highly important to purify the solutions from excess strands and misfolded structures by using methods such as electrophoretic separation, spin-filtering, precipitation by poly(ethylene glycol) (PEG) crowding\textsuperscript{[93]} or rate-zonal centrifugation.\textsuperscript{[94]} In addition, for many uses, the buffer conditions should be carefully optimized to ensure the structural integrity of the DNA objects.\textsuperscript{[95]} However, some DNA objects can maintain their designed shape at very low salt concentrations.\textsuperscript{[96]}

4.2. Combining Top-Down with the Bottom-Up Nanofabrication: Biosensors, Nanopores, Optics, Plasmonics and Molecular Electronics

The structural addressability, molecular scale resolution\textsuperscript{[97],[98]} and the high parallelity that can be achieved using DNA nanostructures could be utilized in novel nanofabrication techniques (combining top-down and bottom-up approaches). For example, DNA origami designs have been utilized as smart lids or gatekeepers on the solid-state nanopores, which could be used in many sensing and sequencing applications.\textsuperscript{[99-101]} Recently, these strategies are adopted for creating versatile and modular membrane-based DNA nanopores.\textsuperscript{[102–106]}

On top of that, DNA origamis can be exploited in creating plasmonic and state-of-the-art optical devices,\textsuperscript{[107],[108]} rulers\textsuperscript{[109]} and templates for optical high-resolution nanoimaging such as DNA-PAINT.\textsuperscript{[110]} Moreover, by combining DNA-based templates with standard top-down lithographic approaches or with modifiable metal nanoparticles and wet chemical growth methods, it could be possible to achieve implementations in molecular electronics and plasmonics and in creating novel integrated circuits (ICs) and fully tunable metamaterials.\textsuperscript{[111–126]}

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4.3. Molecular Computing, Signaling and Robotics

In general, due to its superior self-assembly and programming properties, DNA is an intriguing molecule in molecular computation. DNA molecules and higher-order nanostructures can be used in algorithmic self-assembly and digital computing\(^{127}\) and for example, artificial neural networks can be created at molecular level using DNA strands.\(^{128}\) Moreover, it would be highly interesting to create signaling cascades\(^{129}\) and to probe the mechanical and dynamic properties of DNA nanostructures; for example dynamic robotic-like devices,\(^{130}\) rotational mechanisms\(^{131}\) and information relay systems\(^{132}\) could be created, and it is envisioned that in these systems the shape-complementarity–based designs\(^{78}\) could be further exploited.

4.4. Nanobiomedicine and Characterization of Pivotal Biomolecules

Precisely controlled DNA nano-objects can find interesting applications in characterization and imaging of other biomolecules; for example, they can be used in nuclear magnetic resonance (NMR)-based characterization of membrane proteins\(^{133-135}\) and they can serve as versatile templates for imaging proteins with cryo-electron microscopy\(^{136}\) or for probing e.g. nucleosome interactions.\(^{137}\)

Some of the innovative applications are about to emerge in the medicinal field.\(^{138,139}\) DNA nanostructures can be used as templating enzymatic functions,\(^{140-144}\) as well as vehicles and devices for targeted drug delivery.\(^{145}\) Moreover, rationally designed DNA objects can find applications in detection of antibodies,\(^{146}\) nanomechanical genotyping,\(^{147}\) regulating gene expression\(^{148}\) and in advanced and fully controlled molecular transport.\(^{149}\) Interestingly, DNA structures can be used as smart nanorobots that are able to interact with each other and perform logic operations in living organisms.\(^{150}\) For the genuine combination of nanostructure synthesis and living organisms, there are already ingenious methods to produce custom DNA shapes \textit{in vitro}.\(^{79,151}\)
However, one of the issues in efficient DNA-based drug delivery is the demand for increasing structural integrity and transfection properties, as well as bio- and immunocompatibility of the DNA nanoshapes.\cite{95,138} Recently reported materials that have been used for shielding and coating of DNA origami include virus proteins,\cite{152} lipid membranes,\cite{153} cationic polymers\cite{154-156} and protein-dendron conjugates,\cite{157} which could be used for protecting the structures from endonucleases and degradation in biological environment.

4.5. DNA Nanotechnology Flourishes

Overall, the future seems extremely bright for DNA nanotechnology. The new designs can become key players in rapid progress of various other research fields diverging from materials science to nanobiomedicine. Currently nearly 300 laboratories are working in the field, but as the design methods are becoming more and more accessible for users with different backgrounds, the number of participating groups is ever increasing. Therefore, we strongly believe this development will be highly important for creating novel biomaterials and cutting edge applications.

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DNA nanotechnology flourishes. In this progress report we review the recent progress of structural DNA nanotechnology by presenting versatile design strategies and emerged computer-aided software for design and simulation of the nanoshapes. We envision that the evolution in the field will lead to the development of novel biomaterials and advanced applications.

Keywords: nucleic acids, self-assembly, nanotechnology, computer-aided design, programmable materials

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Evolution of Structural DNA Nanotechnology