Programming structured DNA assemblies to probe biophysical processes

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Abstract

Structural DNA nanotechnology is beginning to emerge as a widely accessible research tool to study diverse biophysical processes. Enabled by the scaffolded DNA origami approach, in which a long single-strand of DNA is weaved completely through a discrete 3D nanoparticle or 2D array, synthetic macromolecular assemblies can now be programmed computationally in a fully automatic manner to interface with biology on the 1–100 nm scale. Here we review the major design and synthesis principles that have enabled the design of specific subclass of scaffolded DNA origami objects called wireframe assemblies, which offer unprecedented control over the nanoscale organization of biomolecules, including concave versus concave geometries, internal versus external functionalization, and full control over biomolecular copy number and nanoscale placement. To highlight the power and versatility of this synthetic approach, we feature its application to three leading areas of biophysical investigation: light-harvesting, RNA structural biology, and immunology, with an outlook towards fundamental insight that may be gained in the coming decade using this unique synthetic structural biology approach to probing molecular biophysics.

Keywords

Nanotechnology; DNA origami; computational design; light harvesting; structural RNA biology; immune recognition

Introduction

The discovery of the double-helical structure of DNA was arguably the most important discovery in biology, if not all of science (45, 163, 166). Driving this landmark discovery was the close interaction between theory and experiment: Quantitative X-ray scattering data generated by Franklin, Wilkins, and colleagues was interpreted theoretically using a structural model of DNA proposed by Watson and Crick. Elucidation of the remarkably simple, elegant structure of DNA, which was confirmed only 20 years later by Rich and colleagues (125), marked the first of several discoveries that enabled the rise of structural DNA nanotechnology to its prominence today, which has similarly been driven by a close interaction between theory and experiment.

Principal among these was the follow-on discovery of the Holliday junction used by cells in DNA recombination and repair, which confirmed that DNA may exist not only as a linear, but also a branched structure (65). This drove the remarkable conception by Ned Seeman in 1982 that Watson-Crick base pairing rules together with multi-way branches may in principle be used to program synthetic DNA oligonucleotides to form complex, custom nanoscale materials (130). Seeman used this concept together with expertise he had gained in DNA synthesis and crystallography while working alongside Rich during his postdoctoral years in order to self-assemble a synthetic, discrete DNA cube composed of 6 DNA strands, with 20 base pairs per cube edge, and an overall cube dimension of 7nm, which arguably represents the birth of the field of structural DNA nanotechnology (25).

Lacking structural rigidity and versatility in its branching capabilities, which limited this initial design strategy from being generalized to more complex geometries, this early architecture consisting of 3-way vertices connected by single helices was soon supplanted by the introduction of the double-crossover (DX) motif. The DX motif displayed a two-fold increase in rigidity and offered in principle the opportunity to program nearly arbitrarily complex nanostructures and arrays (170, 173). Since this time, the field of structural DNA nanotechnology has blossomed to include diverse strategies for programming 2D and 3D materials, which have recently been reviewed comprehensively (85, 117, 131, 132). In the present review, we focus on recent applications of structural DNA nanotechnology to molecular biophysics, and highlight three discrete advances from the past decade that in our view offer immense unexplored opportunity for the field, with a focus on nanoscale light harvesting, RNA structural biology, and immunology.

In the first crucial advance, Paul Rothemund introduced the concept of scaffolded DNA origami in which a long scaffold strand of DNA is folded into a complex, brick-like target shape via hybridization with hundreds of shorter oligonucleotide “staple” strands that hold the structure together via Watson-Crick base pairing (126). This 2D strategy was later generalized to 3D by Shih (35), Yan (138), and colleagues, and offered for the first time the ability to robustly synthesize diverse, discrete, structured DNA assemblies at the megadalton- or 10–100nm-scale for biophysical applications. Importantly, in contrast to tile-based assembly that was previously used to program higher-order structures, scaffolded DNA origami results in the quantitative yield of monodisperse structured DNA assembly products, which can be used much like any discrete macromolecular assembly such as a virus, polymer, or inorganic nanoparticle, with one important distinction: Scaffolded DNA origami is uniquely addressable at any distinct nucleotide position within the megadalton assembly for purposes of molecular functionalization or templating.

In the second major advance, wireframe motifs were conceived of (173) and applied to program nearly arbitrarily complex lattice-like assemblies in 2D and 3D (175). In contrast to brick-like assemblies that are largely limited to acting as pegboards, these assemblies now offer internal as well as external positioning of biomolecules at arbitrary 2D and 3D spatial positions, the facile generation of convex versus concave structured surfaces, access to larger surface area per scaffold length, and are also compatible with a broader range of ionic conditions (160).

In the final major advance that is still ongoing, “top-down” computational design algorithms have generalized the wireframe synthetic approach to programming 2D and 3D assemblies of nearly any shape and size on the 10–100 nm scale fully automatically, now enabling any researcher to participate in the design and application of these structured DNA assemblies to their own research applications of interest, without expertise in DNA origami sequence design (**Figure 1, Sidebars 1 and 2**) (14, 15, 74, 75, 160).

These preceding three major advances of the past decade, namely scaffolded DNA origami, wireframe design,

and full automation of sequence design, together with very recent new strategies for scaffold production using either templated PCR (84, 161) or phage production (118), have enabled the production of sequence- and length-specific DNA scaffolds for use in designing nearly any 2D and 3D target assembly imaginable.

The general workflow employed for wireframe DNA origami design and synthesis is exemplified by the use of the software DAEDALUS (**Figure 1A**)(160). First, the user generates a 3D polyhedron of arbitrary shape as the input to the algorithm. This target polyhedron is then converted into the staple sequences needed to fold the desired scaffold into the target shape through a sequence of fully automated computational steps that include scaffold routing and staple assignment to ensure robust and high-yield folding. Additionally, available algorithms now generate atomic models for both 2D and 3D architectures of arbitrary shape (**Figure 1B**). Once staple sequences have been determined, wireframe origami synthesis proceeds as conventionally performed by thermal annealing using a 10-fold molar excess of staples over scaffold, using folding buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, 12 mM $MgCl_2$ at pH 8.0), which can be exchanged post-folding with PBS or other physiological buffers for applications to molecular biophysics. Purification is then typically performed using either agarose gel electrophoresis, centrifugal concentrators or density gradients, PEG precipitation, or FPLC (24, 88, 118, 139).

Following origami synthesis, predicted atomic models are validated experimentally using atomic force microscopy (AFM) for 2D assemblies or transmission electron microcopy (TEM) for 3D. While technically more challenging, cryogenic-EM offers the possibility of reconstruction to elucidate 3D structure with single-duplex resolution (10, 75, 160). Advanced single-molecule fluorescence imaging using PAINT (73, 76, 149) or Foerster Resonance Energy Transfer (86) can also be applied to characterize nanoscale topology and dynamic intra-molecular distances.

Coarse-grained physics-based models complement structural data and target design models to offer insight into conformational flexibility, mechanical properties, as well as possibly structural deviations from target atomic models. Molecular dynamics (MD) offers the gold standard for atomic-level structural modeling, yet it is limited to a few select groups due to the large computational resources required (86), so coarse-grained modeling approaches using finite elements ((24, 81, 113, 114) and bead-based approaches (145, 150) have played leading roles in the practical design and simulation of DNA origami bricklike and wireframe assemblies in order to elucidate structural details such as overall structural twist and bend (24, 81), as well as vertex geometries and local and global structural rigidity.

Towards functional biophysical applications of DNA origami, conjugation of bioactive molecules at the 3' and 5' ends of staples can be readily performed using either covalently chemistries or simple hybridization to single-stranded 3' or 5' overhangs (**Figure 1C**). Internal oligo modifications can also be utilized, although they are generally more costly unless in-house DNA synthesis is performed. More elaborate DNA nanostructure functionalization strategies have been reviewed in detail recently (54). Because every DNA base in a given scaffolded DNA origami object is known a priori from the target design with single-molecule precision and addressability, it is straightforward to envision applications in which asymmetric positioning of bioactive molecules is utilized. This is particularly powerful in the case of wireframe assemblies that offer both internal and external presentation of 3' and 5' staple ends, which are easily modified to adjust their inward versus outward facing angles within approximately 35 degrees and 0.34 nm accuracy in the axial pitch and rise of the DNA duplex. This powerful capability of spatial control of biomolecular presentation offered by DNA origami combined with the versatility in geometric control offered by wireframe design now offers near full control over our ability to mimic and interrogate biophysical processes using structured presentation of nucleic acids, proteins, and small molecules. Integration of external signals to perform logic operations offers yet another, an entirely distinct arena of temporal control over structured DNA assemblies (174).

These foregoing properties clearly render structured DNA assemblies unique biophysical research tools compared with other classes of nanostructures and nanoparticles including liposomes, dendrimers, polymers, and viral protein mimics (26, 30, 92), which offer large-scale production for in vitro applications, yet lack the ease and versatility of geometric design and asymmetric, orthogonal internal and external functionalization. To highlight these unique capabilities of structured DNA assemblies to probe fundamental questions in molecular biophysics, in the following sections we feature their application to three select, leading research applications

that we anticipate will have transformative impact on their respective fields in the coming decade: nanoscale energy transport and light harvesting, RNA structural biology, and immune receptor signaling.

Biologically-inspired light harvesting

Natural photosynthetic complexes absorb sunlight and transport energy to reaction centers at remarkable efficiencies. This efficient mechanism is due to a number of design principles: directional energy transport and high cross-sectional area for light absorption and transfer, such as in the chlorosome found in the light-harvesting complex of green sulfur bacteria (**Figure 3A**) and phycobilisome antenna found in cyanobacteria (**Figure 3B**), and coherent effects (i.e., electronic and vibrational), which tune the energy transfer pathways and promote charge-separated states (124). These design principles are achieved by embedding photosynthetic chromophores within protein complexes (**Figure 3C and D**) in highly specific arrangements, with respect to both the local protein environment and surrounding chromophores. Considering that the design principles of natural photosynthetic complexes lead to highly efficient energy absorption, transfer across nanometer to micrometer length scales, and energy conversion, can these design principles be effectively mimicked in a biologically-inspired light-harvesting system? DNA nanotechnology offers the potential to mimic the hierarchical and dense organization of chromophores that are typically observed in photosynthetic light-harvesting systems. Specifically, the bottom-up synthesis of biologically-inspired light-harvesting systems with unprecedented single-molecule addressability in the spatial and angular position of dye molecules offers unique opportunity to test long-standing hypotheses in the field of light-harvesting regarding nanoscale energy transport.

Toward this end, several synthetic DNA-dye systems have been built that operate in the weak-coupling regime where incoherent or “hopping” transport is prevalent and can be described with Förster theory. One-dimensional energy transfer through DNA duplexes is the most rudimentary approach to investigate energy transfer. For this purpose, dyes can be incorporated into the DNA scaffold through intercalation (22, 29, 58), groove-binding (6, 77, 133, 146, 172), and covalent attachment (40, 153, 168). Light-harvesting systems that are assembled using intercalation or groove-binding dyes rely on energy migration for transport along the DNA. These dyes can also assist in bridging primary donors to acceptors for donor-bridge-acceptor systems. Another approach to bridge primary donors to acceptors is to have a downhill energy-transfer pathway. This can be achieved by covalently attaching dyes that have cascading energies on the DNA scaffold (60, 99). In contrast to the energy-migration approach where energy transport proceeds through random walks, cascaded energy transfer is unidirectional towards the primary acceptor. This approach can extend downhill energy-transfer distances up to several tens of nanometers with relatively high efficiencies (60).

Scaffolded DNA origami enables the synthesis of larger and modular light-harvesting structures with far-denser dye functionalization patterns compared to earlier approaches such as DNA duplexes and branched DNA structures (171). Because of the designer characteristics of DNA origami, it is possible to discretely control nanoscale energy transport (61). More importantly, DNA origami allows for 3D energy transfer which emulates the energy transfer pathways in natural light-harvesting systems (**Figure 4A**) (37, 112). Long-range energy transport is important in photosynthetic complexes specifically for directionality of energy transfer and for initial capture and funneling of sunlight from the peripheral light-harvesting units. To achieve the greatest quantum efficiency, the interplay between incoherent (i.e., long-range) and coherent (i.e., short-range) energy transfer regimes is essential. More recently, strongly-coupled DNA-dye systems have been studied which operate in the intermediate distance regime seen in photosynthetic systems. Cyanine (20, 97) and squaraine dyes (97) that are covalently attached to DNA as dimers across the DNA duplex show spectroscopic features consistent with Davydov exciton splitting, characteristic of strongly-coupled dye aggregates. Porphyrins, a class of molecular heterocycles involved in photosynthetic light-harvesting, show strong excitonic interactions when covalently attached to DNA (162). Our group has investigated the sequence-selective formation of pseudoisocyanine J-aggregates on DNA duplexes (**Figure 4B and C**) (12, 16). We observed that the exciton delocalization lengths of DNA-templated pseudoisocyanine aggregates determined from superradiant enhancement measurements are comparable to measured exciton delocalization lengths for natural light-harvesting systems (105, 119). Scaffolding J-aggregates using DNA constructs allows for investigation of the effect of static disorder on the overall energy-transfer process (12) and inter-aggregate energy transfer (16) (**Figure 4 and C**).

These recent applications provide a broad overview of the potential of DNA as a scaffolding material for various chromophores, as well as the ability to enable both long-range and short-range energy transfer effects. What remains is whether these various energy-transfer regimes can be integrated as in the photosynthetic antenna, leading to energy transfer efficiencies approaching those of biological systems. In a DNA-dye complex, the

following spatial design principles for light-harvesting are necessary to compete with the efficiencies found in natural systems: (1) designing unidirectional energy-transfer pathways to funnel absorbed photon energy to artificial reaction centers, (2) precise placement of chromophores on a DNA scaffold, and (3) the hierarchical organization of different light-harvesting and energy conversion elements (**Figure 4D**).

Aside from long-range energy transfer mechanisms in natural photosynthesis, the importance of short-range coherent effects between electronic and vibrational states for transfer efficiency remains a point of debate. Electronic coherence occurs when a strong electronic coupling between closely-packed chromophores creates a delocalized manifold of excitonic states, and dynamic motion of the exciton along the chromophores can occur on extremely fast timescales. Electron-vibrational coherence occurs when the electronic coupling between chromophores is similar in magnitude to a vibrational mode, typical of the protein environment surrounding the chromophores. In Engel et al. (39), coherent energy transfer was observed for the first time in a photosynthetic system, the Fenna-Matthews-Olson (FMO) complex, seen as an oscillatory behavior of the excitonic energies via an electron-vibrational coupling with the protein. Likewise, can the environmental modes of the DNA scaffold be used to tune the energy landscape through electronic-vibrational couplings as in protein scaffolds?

Due to their ubiquitous nature in photosynthetic complexes, closely-packed chromophores will be an important design motif for synthetic DNA-dye scaffolded complexes. These DNA-dye complexes can be used both to elucidate the role of quantum coherent states in natural and synthetic systems, and to study the interplay between long-range and short-range energy transfer effects. Single-molecule control of chromophores on DNA scaffolds offers a powerful molecular toolbox to investigate energy-transfer pathways and mimic key structural elements found in natural light-harvesting. The convergence or divergence of synthetic light-harvesting technologies with those of natural light-harvesting systems will be of intense interest in these upcoming studies.

RNA structural biology

In contrast to the highly predictable nature of Watson-Crick base pairing and the resulting structure of the DNA duplex that has enabled the field of structural DNA nanotechnology, RNA is a wholly distinct molecule due to the addition of a 2' ribose hydroxyl that induces a C3'-endo pucker. This results in RNA taking on an A-form double helix, as opposed to the B-form double helix adopted by DNA. In addition, RNA uses uracil bases rather than the thymine bases found in DNA, and RNA is typically single-stranded in the cell. These features combine to make RNA a highly versatile molecule, endowed with immense conformational variability. This renders the prediction of RNA secondary and particularly tertiary structure from sequence or primary structure challenging, with a major need for new tools to determine the 3D structural and dynamical features of long RNAs (103, 137). RNA's chemical differences with respect to DNA also result in diverse catalytic activity and chemical reactivity, which are exploited in applications of RNA synthetic biology (69).

The cell takes advantage of secondary and tertiary RNA structures to regulate translation and chemical activity of naturally occurring ribozymes (18, 122, 134). Viral RNA genomes, including those of Ebola and HIV, exhibit numerous unique structural motifs due to their large mutation space and selective pressure towards maintaining small genomic sizes. Detailed knowledge of these genomic structures may generate new targets for anti-viral drug development (96)—analogous to the discovery of structure-activity relationships from understanding mechanisms of antibiotic-binding to the ribosome (167). Insights into RNA tertiary structure should also improve design approach for RNA aptamers (38, 159) and ribozymes for biological engineering applications (72, 78, 111, 136).

Indeed, secondary and tertiary structures have already enabled numerous advances in structural RNA nanotechnology. Instrumental work includes the generation of nanoparticles composed of tectonics, or structural subunits such as tRNAs and using naturally-occurring junctions (2, 31, 135, 142, 165). Leveraging these native structures with engineered connecting mechanisms enables scalable production of RNA structures, achieving user-defined nanoparticles. Additionally, employing native RNA interactions such as validated kissing loops or programmed paranemic crossovers allows RNA-based structures to fold from a single strand (1, 50, 57, 82), with applications in from molecular capture and sensing in cells to therapeutic applications (64).

Despite the importance of RNA tertiary structure, little is known about global 3D RNA folds, their stability, and dynamic interconversions including mediation by RNA-binding proteins. This is in part due to the lack of 3D structural characterization tools, with methods to probe local secondary structures and higher-order tertiary interactions in large RNAs still actively being explored (103, 137). Physics-based and homology-modeling computational algorithms along with chemical probing have been successful in secondary structure determination, but tertiary structure prediction and measurement techniques remains largely absent (7, 32, 43, 63, 102, 103, 178) (SHAPE)(93, 101, 143, 164) (123). Limitations in the applicability of conventional structure-determination strategies to RNAs largely reside in difficulties in applying them to long RNAs due to their high molecular weight and conformational heterogeneity. In certain well-ordered or short RNAs, several techniques have applied, including X-ray crystallography (177) (56, 79, 157) (11, 128, 169), small angle x-ray scattering (28, 41), single-molecule fluorescence resonance energy transfer (31, 116, 156), and nuclear magnetic resonance (80, 176). Finally, cryo-EM offers a viable alternative for high-throughput RNA structure determination with high resolution (49); however, this technique relies on large numbers of uniform particles to get accurate class averages.

One of the greatest achievements in RNA structural biology is the high-resolution structure of the ribosome (**Figure 5A**). Through decades of research, we know the large subunit of the ribosome is responsible for the peptide transfer occurring at the peptidyl-transferase center (PTC), coordinating the P-site tRNA and catalyzing the transfer of the growing peptide to the amino acid on the A-site tRNA, before ratcheting the tRNAs to the next sites in a cyclical, coordinated dance that has been excellently reviewed (148). Atomic-resolution structures of the ribosome and folding intermediates (**Figure 5B**), have allowed for a greater understanding of the folding and reactivity of the PTC (**Figure 5B**). Attaining these high-resolution structures is due in part to the conformational stabilization of the RNA by protein interactions, a principle that can be regarded as nature's "RNA origami" and might inform novel strategies in RNA structural biology.

Toward this end, we speculate that structured DNA nanoparticles may present one viable solution. Specifically, DNA origami may be used to coordinate RNAs to elucidate tertiary structure, probe population dynamics, test protein-RNA interactions, and study RNA domain folding and maturation (**Figure 5C**). Furthering structural biology has long been a goal of DNA nanotechnology as originally proposed by Ned Seeman, and recently, structured DNA origami coordinating proteins (48, 147, 154) has advanced this goal. Coordination of RNA using engineered loops as part of the staple or scaffold sequence allows for the organization of a molecule within designed origami with nanometer spatial precision. This strategy is appealing for several reasons; in particular, tuning the avidity of the DNA origami-RNA interaction can segment populations of distinct native RNA conformations for dynamic, flexible RNA molecules. Restricting the RNA conformation via such a binding interaction can enable cryo-EM elucidation of the RNA structure bound to the DNA “cage”, allowing for high-throughput structural and biochemical characterization at the 1 to10 kilobase-scale. Integrating these structural studies with single-molecule FRET may reveal conformational heterogeneity and sub-states that are otherwise impossible to resolve using crystallography or solution- or surface-based structural studies that interrogate RNAs in their isolated, native state.

Further, the ability to specifically fix complex RNAs to nucleic acid origamis in precise orientations opens transformative areas of research, such as the potential recovery of PTC ribozyme activity outside of the ribosome that otherwise requires coordination by ribosomal proteins (**Figure 5C**), an as-of-yet unsolved problem. Application of structured DNA nanoparticles may also assist in this regard, enhancing our ability to engineer orthogonal translation systems (107, 109), provide new tools to investigate RNA modification biochemistry (55, 120, 121) and ribosome assembly and folding (152), while enabling new tools in minimal synthetic translation systems (44, 141). Due to the exquisite 3D assembly of particles with specific, programmed interaction sites, the field of nucleic acid nanotechnology may interact with, and ultimately impact, the RNA structural biology field with relevance to drug discovery and possibly even the origin of life.

Immune receptor signaling

Cell membrane receptors facilitate the transport of information and material through the plasma membrane, allowing cells to interact with their external environments and respond to stimuli. Amongst them, immune receptors represent an important class of receptors that includes the innate immune pathogen recognition receptors as well as adaptive immune system highly specific antigen receptors on the surface of T and B cells (**Figure 6A**). These receptors have evolved methods to differentially sense variety of antigens and pathogens associated macromolecular patterns including polysaccharides, lipids, and protein/peptide epitopes and their byproducts over self-epitopes (46, 70). They sense features of ligand organization on the pathogen surface that reflect the characteristics of targets and determine downstream behavior, such as the number and stoichiometry of ligands(62) as well as their 3D organization (19, 27). The coordinated signaling of these receptors help distinguish self from foreign pathogens (100), and pathogens from commensal microbes (3). Understanding how these features impact immune receptor function gives insight into the factors that drive cellular responses during allergy (47, 127), autoimmune disease (98), and cancer (115, 129). Further, better understanding of immune cell surface receptor systems will directly impact the design and development of more efficient vaccines and cancer immunotherapies.

Immune receptors are expressed on a variety of cell types, including non-immune cells, and perform their function both on the surface of the cell and within internal compartments. Often, cellular sensing of pathogen-associated molecular patterns and antigen happens in the context of a specialized tissue compartment such as the lymph node, involves part or whole of pathogen surfaces, coordinates with fixed complement, and sometimes occurs in the context of cell-cell synaptic adhesions. Immune receptor signaling is commonly regulated by co-receptors and cross-talk between transmembrane receptors which sense environmental cues in a spatially dependent manner (23, 66, 71). Further, coordination between immune cell surface receptors has been shown to be involved in HIV entry into T-cell and other viral infections (89). These complex factors can influence the behavior and fate of the cell downstream from receptor signaling events, however, understanding how clustering of and coordination between adaptive immune receptors (**Figure 6A**) influences signaling and cellular fate remains challenging (59).

Materials that reproduce features of antigens and pathogen associated molecular patterns allow for certain contextual features of immune receptor activation to be reproduced in vitro, facilitating the understanding of their relative contributions (**Figure 6B**). These materials include supported lipid bilayers with tethered antigens (19) which facilitate the study of surface tethered antigen and can be extended to systems mimicking antigen presenting cells. Lipid vesicles (21) as well as patterned surfaces (95) allowing for T cell receptor ligand clustering and partitioning of TCR ligands to study the minimum number of ligands needed to trigger TCR clustering and activation (**Figure 6B**) (19, 33, 95) or containing ligand-receptor pairs have also been used to study the effects of cell-cell adhesion during antigen receptor activation. In addition, multivalent chemical polymers (13), synthetic antigen peptides (8), and engineered phage (83) and viral (9) systems have helped shed light on the effect of affinity and multivalency during antigen receptor activation.

Further, receptor colligation of receptors and co-receptors has highlighted the cooperativity between immune receptors (23, 67), and trivalent presentation of TLR9, TLR7, and TLR4 agonists demonstrated coordination between distinct TLRs (155). Many of these systems display limitations with regard to problems controlling rigidity, stoichiometry, orthogonality, and placement of ligands and antigens. DNA-based materials including DNA origami are able to meet these demands as they offer high-degree of control over antigen placement, stoichiometric control and design of antigen presentation, and control of rigidity through well characterized DNA scaffolds (36, 126, 160) (**Figure 6C**) and offer a high degree of biocompatibility (151).

To date, while only several examples have been published, the important potential for structured DNA assemblies to investigate geometrical requirements of immune receptor signaling and function has been demonstrated. For example, trimeric DNA duplexes were used to template dinitrophenyl (DNP) antigens in a mast cell-IgE system having specificity against the DNP monomers. By increasing the spacer distance between the DNP monomers, the authors showed a dependence of IgE receptor signaling on inter-antigen distance, suggesting cooperativity between the receptors (144).

Other examples with non-immune receptors also highlight the potential of the DNA nanostructures for ligand presentation. Recently, a large scaffolded DNA origami structure was used to present dimers of ephrin ligands to MDA-MB-231 cells expressing the ephrin receptor EphA2 (**Figure 6B**). Here, the authors compare monomeric, modified rod-like nanoparticle to two different dimers with either 40 nm or 100 nm inter-ligand distances. These results showed a signaling response dependent on the inter-ligand spacing in the DNA origami system, and also establish that dimer distances greater than 100 nm are quantitatively equal to monomeric binding, supporting cooperativity between the receptors only at short distances (140). In separate work, the Niemeyer group used a surface-based DNA origami system to pattern epidermal growth factor receptor (EGFR) ligands and explored the effects of inter-ligand distance and ligand stoichiometry. These results suggested that placing EGFR receptor 5 nm apart yields lower cellular responses than ligands with inter-antigen spacing greater than 30 nm (4).

Generally, these studies suggest that DNA-based spatial control over antigen and ligand may be used to understand the spatial effects of receptor cooperativity during receptor engagement, and highlight important differences in how these receptor systems respond to ligand patterning. In future work, it will be important to consider the use of assays to read out the organization of ligands on DNA nanoparticles and the organization of receptors following binding. DNA-PAINT has already proved very useful for this task, and has been used to confirm the incorporation and accessibility of single strand target DNA presented on DNA origami (149). Structured DNA has also been used to understand how stoichiometry impacts Toll-like receptor signaling. Indeed, Immune cells are particularly sensitive to CpG DNA sequences, which are highly abundant in bacterial genomes, and are recognized by the pattern recognition receptor Toll-Like Receptor 9 (TLR9) as well as other pattern recognition receptors (17, 104). Comparing the immunostimulatory response of DNA nanoribbons functionalized with CpG to duplex and monomeric presentation of CpG showed a marked increase in immune stimulation of the highly repetitive nanoribbon(110). Moreover, similar structures have already been used in vivo as vaccine platforms (91) as well as mechanisms for modulating immune response (68).

DNA materials have only just begun shed light on the molecular requirements of immune cell surface receptor function. To date, studies have focused largely on CpG-modified DNA nanomaterials due to the facile attachment of oligonucleotides through base-pairing interactions. Functionalization of DNA origami with antigens will facilitate the study of a broader range of immune receptors, where functionalization will rely upon a variety of chemical techniques to attach peptide and protein ligands to preserve antigen and ligand function following attachment to scaffolded DNA origami objects (**Figure 6C**). These features will further add to the growing body of knowledge on the cooperativity between distinct surface receptors, serving as tools to study the complex network of signal regulation and pathogen discrimination in immune cells. Further, improved tools for design and synthesis of DNA origami will aid efforts to understand the dependence of receptor responses on inter-antigen spacing and other geometric features, where the facile design of customizable DNA origami will allow for full site-addressability of ligands and antigens on DNA origami of arbitrary shape and size.

Conclusions and Perspectives

Controlling the spatial organization of bioactive molecules using structured DNA assemblies offers tremendous potential for molecular and cellular biophysical research. Unprecedented control over the stoichiometric loading, geometric configuration, and mechanical properties of scaffolded DNA origami offers researchers the ability to mimic and manipulate macromolecular organization on the 1–100 nm scale in order to advance our mechanistic understanding of biological structure and function in areas ranging from light harvesting to immunology to RNA structural biology.

In biologically-inspired light harvesting, early approaches employed linear and branched DNA duplexes to organize chromophores to control directional energy transport and enhance absorption efficiency. In comparison, DNA origami has recently enabled the synthesis of considerably more elaborate, modular light-harvesting structures that mimic important aspects of natural photosynthetic systems (37, 61, 171). The continued exploration of these biologically-inspired systems will be further enabled by advances in structural DNA nanotechnology that leverage 2D and 3D wireframe designs, including the ability to construct hierarchical chromophore assemblies that integrate long-range and short-range transfer mechanisms to reveal fundamental insight into natural mechanisms of nanoscale energy transport and light-harvesting (12, 16).

Similarly, wireframe DNA origami offers a uniquely powerful tool to addressing challenging questions in structural RNA biology. Structured DNA nanoparticles with convex exteriors and concave, hollow interiors can be employed to stabilize flexible viral RNAs by presenting selective single-stranded overhang motifs that are complementary to single-stranded RNA regions revealed using chemical footprinting. This approach may be used to stabilize specific tertiary conformations that can subsequently be elucidated at high resolution using single-particle cryo-EM. Incorporation of RNA-binding proteins and chemical modifications can be used to test their impact on structural heterogeneity. Alternatively, the precisely controlled 3D geometry of structured DNA assemblies can be used to scaffold RNA motifs to test hypotheses surrounding the structural origins of their catalytic activity, such as in scaffolding catalytic RNAs to synthesize artificial PTCs, with potential to provide fundamental insights into ribosome evolution and function.

Finally, controlling the 1D, 2D, and 3D spatial organization of immunogens or PAMPs on the 1–100 nm scale offers important potential to reveal mechanisms of immune cell recognition, activation, and signaling. Unlike natural or synthetic viral constructs for immunogen presentation, structured DNA assemblies offer full control over copy number, spacing, and the dimensionality of presentation, providing immense room to study how these independent structural features of pathogens have evolved to interact with immune cells. Basic questions surrounding immune receptor clustering and kinetic segregation of phosphatases and kinases that are thought to depend on these structural features can now be tested directly using DNA origami.

The emergence of fully automated, top-down design strategies for 2D and 3D wireframe structures over the last few years together with simplified protocols for molecular functionalization and scaffold synthesis should help disseminate more broadly the application of scaffolded DNA origami to diverse research areas in molecular biophysics and structural biology. In addition to the several highlighted areas of focus in this article, namely light-harvesting, structural RNA biology, and immunology, we anticipate significant impact in related areas as DNA origami continues to become more accessible, and its unique power to address fundamental biological questions continues to be demonstrated.

While this article focused largely on static structural assemblies, DNA nanotechnology offers equal if not more room for transformative impact using its ability to sense external signals and respond using logical operations (34, 87). These dynamic operations offer additional spatial-temporal control, for example in the induction of immune signaling, or ligand-binding. Further, the mechanical properties of origami can be controlled passively as well as dynamically to sense and transduce mechanical stress in situ, offering additional opportunity for studying membrane biophysics and signaling, as well as numerous other areas of molecular biophysics.

Given the very recent rise of computational tools to perform fully automated sequence design, as well as predict 2D and 3D structure and mechanical properties, substantial research is now needed to further our understanding of these properties experimentally. While Seeman's landmark conception of structural DNA nanotechnology took

place over 30 years ago, and Rothemund's conception of scaffolded DNA origami took place over 10 years ago, major computational advances in fully automated, top-down sequence design and controlled scaffold production have only occurred recently, and we therefore believe that structural DNA nanotechnology remains in its infancy as this still nascent field expands its reach to molecular and cellular biophysicists worldwide, "with plenty of room at the bottom" (42).

Summary Points

- DNA nanotechnology offers the ability to spatially organize or interface with biomolecules in 1D, 2D, and 3D. Unprecedented geometric control at the 1–100 nm scale in combination with single-molecule addressability and programmability renders these nanostructures powerful tools for biophysical research.

- Recent advances in the automated, top-down design of 2D and 3D wireframe architectures are democratizing scaffolded DNA origami. Non-experts working in biological research and related fields can now leverage DNA nanostructures to manipulate or mimic fundamental biological structures and related processes.

- We highlight intriguing applications of wireframe DNA origami assemblies to biologically-inspired light harvesting, RNA biology, and immunology, for which we anticipate substantial impact in the coming decade.

- Photosynthetic systems leverage the hierarchical and dense organization of dyes to enable the absorption of sunlight and directional energy transfer at remarkable efficiency. Both long-range and short-range energy transfer mechanisms contribute to this efficiency. By controlling the organization of dyes on the 1–100 nm scale, DNA origami offers unique opportunities to integrate both mechanisms for investigating or mimicking fundamental aspects of light harvesting.

- The elucidation of RNA tertiary structures remains challenging, predominantly due to RNA conformational flexibility and molecular weight. DNA origami-based wireframe assemblies can be used to stabilize conformational states and thereby facilitate structure elucidation by cryoEM. Moreover, we envision that these assemblies might enable the organization of RNAs to generate artificial ribozymes, potentially mimicking the PTC of the ribosome.

- The clustering of immune receptors, the recruitment of co-receptors or the kinetic segregation of phosphatases and kinases represent fundamental processes governing immune recognition. The organization of antigens or PAMPs at the 1–100 nm scale using DNA origami is ideally suited to systematically evaluate these processes and to infer their impact on the induction of endocytosis and immune cell signaling.

Future Issues

- It will be essential to further decrease the cost of DNA nanotechnology.
- Strategies for the combinatorial synthesis of functionalized DNA nanoparticle libraries will enable high-throughput synthesis and downstream applications.
- Mimicking the local chemical environments of dyes and biomolecules typically embedded in protein and lipid environments may be essential for native biophysical studies.
- Incorporating physiologically relevant conditions into RNA structural studies will be essential to understanding native folds and conformational states.
- In vivo applications of structured DNA assemblies such as vaccines will require use of modified and functionalized oligos to stabilize and passivate them.

Sidebars

Sidebar 1. Early computational approaches to DNA origami design

A major bottleneck that has limited the widespread application of scaffolded DNA origami objects to biophysical studies is the complexity of designing the hundreds of staple strands needed to hybridize and fold the 7 kilobase scaffold strand such that it adopts the target 3D structure of interest. The computational design tool caDNAno has played a major role in overcoming this barrier for brick-like assemblies by enabling semi-automated staple design (36), which is typically combined with the 3D structure-prediction software CanDo to optimize staple crossovers prior to synthesis (24, 81, 114) (**Figure 2**). However, scaffold routing and staple design for wireframe assemblies is prohibitively complex using caDNAno, even for experts, requiring fully automated scaffold and staple design algorithms.

Sidebar 2. Automated, top down design of DNA origami wireframe assemblies

Manual scaffold routing and staple design for wireframe assemblies is prohibitively complex. Hence, the "top-down" design tools DAEDALUS, PERDIX, TALOS, and vHelix have been developed to perform automated scaffold routing and oligo staple sequence design based only on a target geometry, without any human assistance in the case of the first three (**Figure 2**). DAEDALUS solves the scaffold routing and staple design problem fully automatically for any closed, 3D nanoparticle surface using solely DX-based edges. TALOS also renders any closed, 3D nanoparticle wireframe surface but using considerably more rigid honeycomb edges, thereby also increasing the scaffold length and nanoparticle molecular weight (75). PERDIX performs fully automated scaffold routing and staple sequence design for any free-form 2D geometry, also using DX-based edges (75). vHelix is an alternative for the design of 2D and 3D wireframe objects, but it utilizes hybrid single-duplex- and DX-edges connecting odd-degree vertices together with physics-based modeling in order to solve the scaffold routing and staple design problem by iteratively adjusting wireframe edge lengths empirically (14, 15), whereas DAEDALUS, TALOS, and PERDIX are all fully automatic and use exclusively DX- or honeycomb-based edges for enhanced structural integrity.

Figure Legends

Figure 1. Design and synthesis of DNA origami nanostructures for biophysical research.

A. Workflow for designing DNA origami objects using DAEDALUS. Initially, a target polyhedron is used as input to the algorithm. DAEDALUS automatically routes the scaffold through every edge and designs the staples needed to fold the object. An atomic model is also generated to allow researchers to visualize the object and design biomolecular functionalizations. Using the computationally designed set of oligo sequences, the DNA nanoparticles is self-assembled via thermal annealing with an excess of staples over scaffold, which is typically based on the M13 phage or a synthetic sequence. Structural characterization is typically performed using agarose gel electrophoresis, AFM, and TEM or cryoEM. Adapted with permission from (160).
B. A vast number of different wireframe architectures of arbitrary shape can now be designed from the top down using the software packages DAEDALUS and PERDIX. Adapted with permission from (75, 160).
C. Functionalization patterns on wireframe objects can be controlled with single-molecule precision. Various strategies for the functionalization of staples at 3' or 5' locations have been established including hybridization of single-stranded overhangs with LNA or PNA sequences and direct chemical modifications.

Figure 2. Automated, top-down design strategies for wireframe scaffolded DNA origami nanostructures.

Following the semi-automated design and visualization software program caDNAno for bricklike scaffolded DNA origami assemblies, several approaches for the fully automated, top-down design of scaffolded DNA origami objects have been reported recently, including DAEDALUS, PERDIX, TALOS, and vHelix. Sequence design results from caDNAno can be imported to the software CanDo to predict 3D solution structures (left; adapted with permission from (35, 36, 81)). vHelix enables the semi-automated, top-down design of both 2D and 3D wireframe objects using predominantly single-helix edge architectures (second from left; adapted with permission from (14, 15)). In contrast, DAEDALUS, PERDIX, and TALOS perform user-free, fully-automated design of 2D and 3D wireframe structures of arbitrary shape and enhanced structural rigidity. Both DAEDALUS (middle; adapted from (160)) and PERDIX (second from right; adapted with permission from (75)) use DX edges exclusively whereas TALOS employs honeycomb edges exclusively (right; adapted with permission from (75))

Figure 3. Photosynthetic light-harvesting.

A. Schematic representation of a light-harvesting complex in the green sulfur bacterium. Chlorosomes have high absorption cross-sections that harvest light and transfer energy to the Fenna-Matthews-Olson (FMO) complex through the baseplate. Absorbed energy is then transferred to reaction centers.
B. Illustrative representation of the phycobilisome light-harvesting antenna. Bilin-containing proteins, phycoerythrin, phycocyanin, and allophycocyanin, absorb light in the green, orange, and red regions of the visible light spectrum. Energy transfer cascade from the phycoerythrin to allophycocyanin via phycocyanin funnels the energy to Photosystem I and II reaction centers (51, 52, 106).
C. Protein scaffolds in the FMO control the spatial organization and influence the site energies of bacteriochlorophyll pigments (158) (PDB ID: 3EOJ).
D. LH2 light-harvesting complex in purple bacteria has a characteristic circular structure which contains the B800 and B850 rings. Proteins control the spatial organization of bacteriochlorophyll pigments in the B800 and B850 rings. These different organizations lead to exciton delocalization (105) and very fast energy transfer (94).

Figure 4. Biologically-inspired artificial light-harvesting.

A. 3D energy transfer on DNA duplex bundles. DNA nanostructures enable the controlled variation of the distances and numbers of dyes scaffolded in order to investigate directed energy transfer. Adapted with permission from (37).

B. The energy-transfer efficiency of DNA-templated PIC J-aggregates to Alexa Fluor 647 decreases with the length of DNA template due to static disorder. Adapted with permission from (12).
C. The sequence-selectivity of PIC aggregation presents an opportunity to create higher-order excitonic circuits to understand the dynamics of inter-aggregate energy transfer. Adapted with permission from (16).
D. DNA nanostructures can be leveraged as designer nanoscale scaffolds to understand energy funneling and directed energy transport mechanisms that are typically found in natural light-harvesting systems. The ability to program light-harvesting structures across different length scales using DNA—from the nanoscale distance of dyes to the microscale organization of light-harvesting DNA constructs (90)—provides a path towards mimicking photosynthesis. Adapted with permission from (90).

Figure 5. RNA structure and capturing.

A. The structure of the ribosome has been solved to atomic resolution (PDB ID: 1GIY and 1JGO; top), which has yielded biological insights into ribosome activity (bottom; adapted with permission from (5)), including coordination of tRNAs in the peptidyl-transferase center (PTC).
B. Folding intermediates of the ribosome show late assembly of an active PTC (adapted with permission from (108)), with the active conformation in the bottom panel (PDB ID: 4V6F).
C. Nucleic acid origami of different sizes and shapes can be used to coordinate RNA structures, modeled for size comparison here with a tRNA (PDB ID: 1WZ2), RNase P (PDB ID: 3Q1R), group II intron (PDB ID: 4DS6), and the 50S subunit of a ribosome (PDB ID: 2WWQ), for applications in structural RNA biology (top) and catalytic activity (bottom) with a hypothetical minimized PTC modeled into an octahedron (PDB ID: 1GIY). The P-site tRNA is shown in green and the A-site tRNA is shown in red.

Figure 6. Templated antigen and ligand systems can be used to query the behavior of surface receptor systems.

A. Schematic of T cell receptor and B cell receptor activation by pathogens showing importance of the nanoscale organization of receptors to trigger immune response.
B. Effects of T cell receptor ligands density and cluster size on T cell receptors activation (left; adapted with permission (19)). A minimum number of T cell receptor ligand are needed to induce T cell synapse formation (middle, adapted with permission from (95)). DNA origami has been used to present ligands at specific distance to study receptor activation (right, adapted with permission from (140))
C. DNA nanoparticles can be designed to query the effects of distance, stoichiometry, dimensionality, and multiplexing through site-specific control of modifications. DNA origami can be used for surface patterning to study antigen pre-cluster distance effect for immune receptor activation. Adapted with permission from (53).

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