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Review

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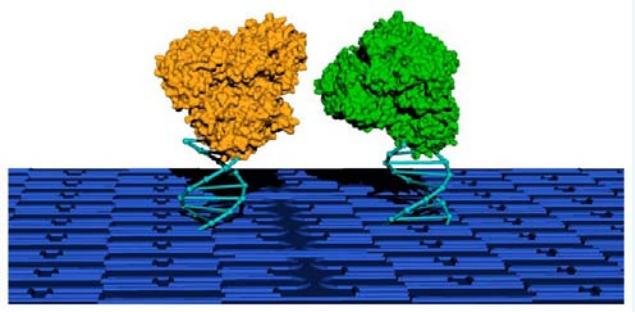
DNA Nanostructures as Programmable Biomolecular Scaffolds

Yuhe R. Yang, Yan Liu, and Hao Yan*

Center for Molecular Design and Biomimetics, and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287, United States

ABSTRACT: This Review focuses on how to use DNA nanostructures as scaffolds to organize biological molecules. First, we introduce the use of structural DNA nanotechnology to engineer rationally designed nanostructures. Second, we survey approaches used to generate protein−DNA conjugates. Third, we discuss studies exploring DNA scaffolds to create DNA nanodevices to analyze protein structures, to engineer enzyme pathways, to create artificial light-harvesting systems, and to generate nanomachines in vitro and in vivo. Future challenges and perspectives of using DNA nanostructures as programmable biomolecular scaffolds are addressed at the end.



1. INTRODUCTION

Nature is a master at organizing biomolecules in all intracellular processes, and researchers have conducted extensive research to understand the way enzymes interact with each other through spatial and orientation positioning, substrate channeling, compartmentalization, and more. In this Review, we address the recent developments of DNA nanostructures in applications of ordering assembly of biomolecules such as enzymes and related cofactors. We discuss the current stage of DNA nanotechnology, survey approaches to generate protein−DNA conjugates, analyze studies of effects of spatial arrangements of biomolecules, and discuss future applications.

B-form double-stranded (ds) DNA has a well-defined structure with a helical repeat of 3.4 nm and diameter of 2 nm. Other than the simple dsDNA helices, more complex structures are realized by rational design of immobile Holliday Junctions, which was first brought up by Seeman.[1] Numerous kinds of DNA nanostructures have subsequently been developed, including double-crossover (DX) DNA tiles,[2] triple-crossover (TX) tiles,[3] 4 × 4 tiles,[4] and three-point-star tiles.[5] The complexity of DNA nanostructures have increased even further by connecting DNA tiles in different ways using sticky ends, resulting in unique higher order nanostructures such as 2D lattices,[4,6−8] nanotubes,[9,10] and more complicated 3D structures such as polyhedra[5,11−13] and crystals formed by tensegrity triangle DNA tiles[14] (Figure 1A).

Scaffolded DNA origami was developed to construct spatially addressable, finite sized DNA nanostructures.[15] In this method, a long, circular single-stranded genome DNA was used as a scaffold to fold into various geometrical shapes with the help of hundreds of short oligonucleotides serving as "staples" strands. With this technique, arbitrarily shaped 2D nanostructures can be constructed with high yield. DNA origami technique was further developed to design[16] and create compact 3D DNA nanostructures[17−20] and elaborate architectures with complex curvatures[21] and gridiron like structures[22] (Figure 1B). As the complexity of DNA nanostructures increases, methods need to be exploited[23−27] to reveal their finer structural details.

More recently, a scaffold-free assembly strategy called single-stranded DNA tiles (SST) was developed by the Yin group[28] (Figure 1C). In this case, ssDNA was used as bricks to assemble or engrave into differently shaped nanostructures. This method has also been employed to grow DNA lattices with controlled depth[29] (Figure 1D).

DNA nanostructures of high programmability and complexity provide excellent scaffolds to arrange multiple molecular/macromolecular components at nanometer scale to construct interactive biomolecular complexes and networks. Due to the sequence specificity at different positions of the DNA origami nanostructures, spatially addressable molecular pegboard with a resolution of several nm (less than 10 nm) can be achieved. Peptides[30] and proteins[31] can be ordered with well controlled intermolecular distances and relative ratios. So far, scientists have studied distance-dependent molecular interactions, substrate channeling, and compartmentalization effects using DNA nanostructures as structural templates. DNA nanostructures can be used to build nanodevices ranging from in vitro small molecule biosensing to sophisticated in vivo therapeutic drug delivery systems and multienzyme networks.

2. PROTEIN−DNA CONJUGATION METHOD

The first technical challenge of building enzyme−DNA complexes is developing conjugation methods to link protein-of-interest and synthetic oligonucleotides. Because DNA molecules themselves display limited chemical functionality, scientists have developed different kinds of biomolecule−DNA coupling methods. The facile chemical modification of nucleic acids with various functional groups on ends or phosphate

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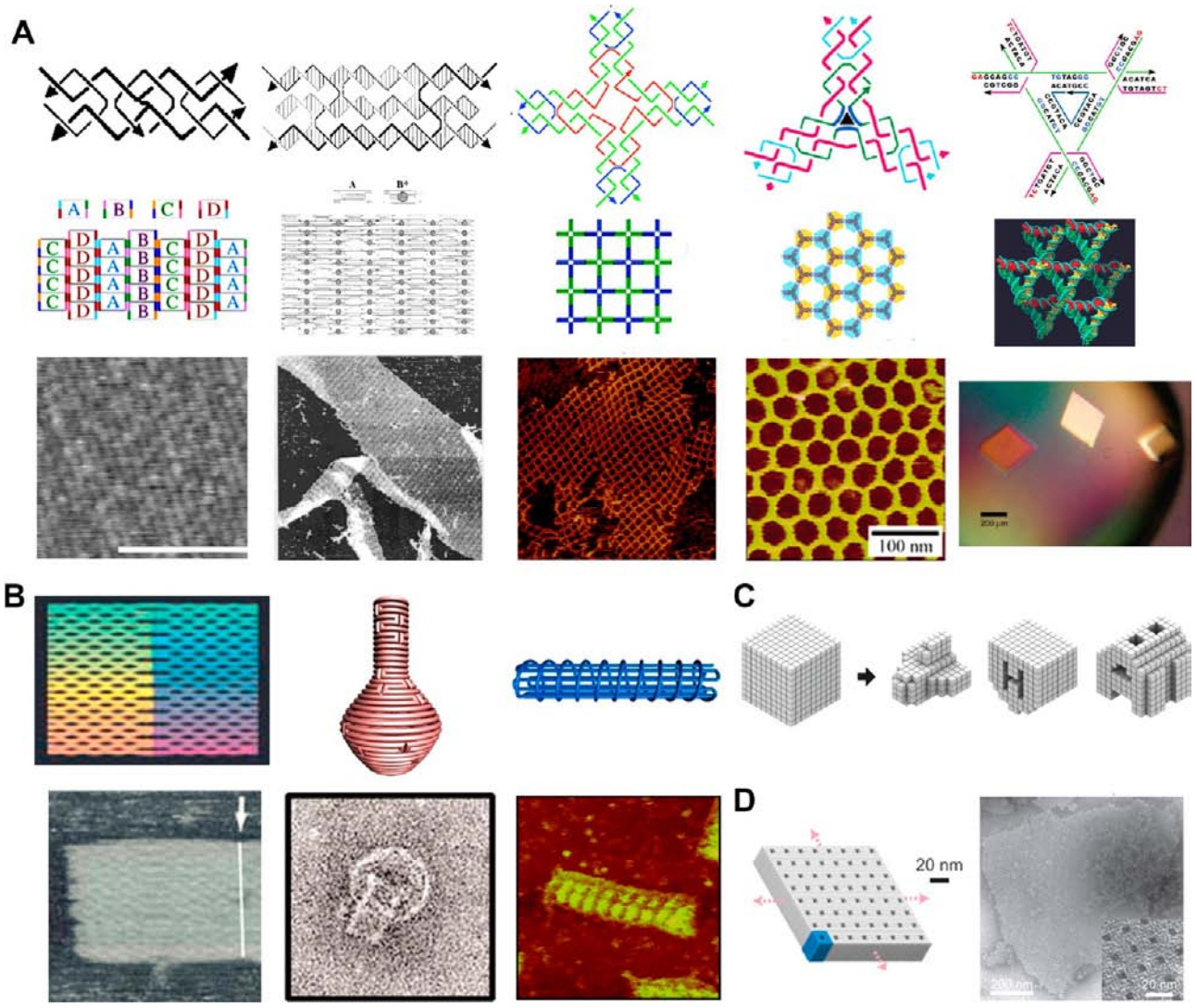

Figure 1. Examples of rationally designed DNA nanostructures. (A) DNA tile based periodic 2D lattices and 3D crystal (top, structures of motifs; middle, cartoon of 2D and 3D assembly patterns; bottom, AFM images of 2D lattices and optical image of 3D crystal). The building blocks (from left to right) include double-crossover (DX) DNA tile,[2] triple-crossover (TX) tile,[3] 4 × 4 tile,[4] three-point-star tile,[5] and tensegrity triangle tile.[14] (B) Schematics (top) and AFM/TEM images (bottom) of DNA origami nanostructures in 2D and 3D shapes, including (from left to right) a 2D rectangular DNA origami,[15] a 3D DNA nanoflask structure with complex curvature,[21] and a 3D screw like DNA gridiron structure.[22] (C) Schematics of multiple single-stranded tile based 3D structures engraved from a cube.[28] (D) Schematics (left) and TEM images (right) of single-stranded tile based 2D crystals with controlled depth.[29] Images reproduced with permission: (A) left to right: ref 2, copyright (2003) Nature publishing group; ref 3, copyright (2000) American Chemical Society; ref 4, copyright 2003 American Association for the Advancement of Science; ref 5, copyright (2008) Nature publishing group; ref 14, copyright (2009) Nature publishing group; (B) left to right: ref 15, copyright (2006) Nature publishing group; ref 21, copyright (2011) American Association for the Advancement of Science; ref 22, copyright (2013) American Association for the Advancement of Science; (C) ref 28, copyright (2012) American Association for the Advancement of Science; (D) ref 29, copyright (2014) Nature publishing group.

backbones enables functionalization of DNA nanostructures. Single-stranded DNA is easily functionalized, and oligonucleotides are normally stable during the organic synthesis process.[32] Most of the derivatives can be prepared by automated solid-phase synthesis and are commercially available. Functional groups include amine, thiol, azide, and so forth.

In contrast, protein modification requires more caution. All reactions have to be done in mild conditions so that the enzymes remain active, especially for functional proteins. Numerous methods for protein−DNA conjugation have been explored, including noncovalent/covalent and non-site-specific/site-specific methods.[33−35] Each method has different advantages and disadvantages, so the choice depends on the applications. For more general conjugation methods that do not require protein engineering, control of the conjugation site and stoichiometry is challenging. This is due to multiple lysine and cysteine residues displayed on the protein surface. In contrast, methods involving protein engineering provide site-specificity and exact stoichiometry, while protein engineering could be problematic and sometimes not successful due to insolubility or misfolding of the engineered protein. Here we categorize protein−DNA conjugation approaches into three groups: noncovalent binding, covalent binding without protein engineering (nonsite specific), and covalent binding with protein engineering (site-specific).

2.1. Noncovalent Site-Specific Conjugation Method. *a. Affinity Tags.* The most predominantly studied method for coupling proteins and DNA are through the noncovalent

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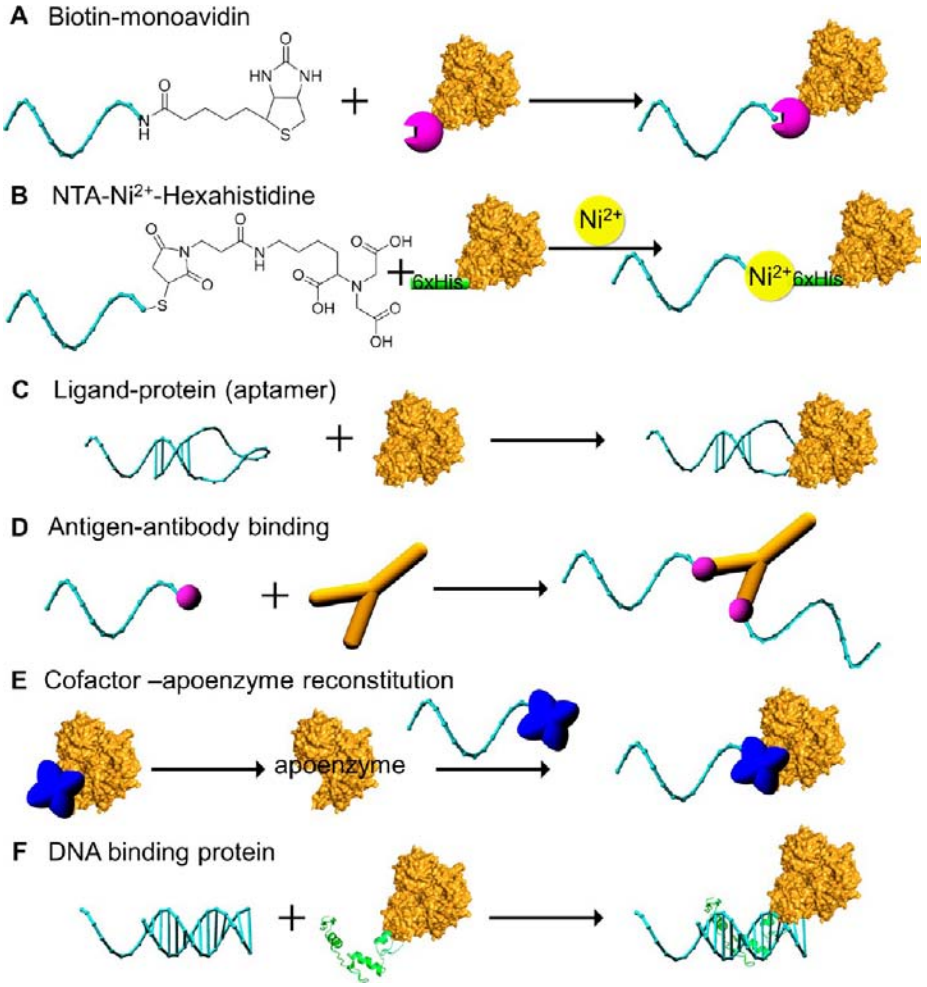
Scheme 1. Reaction Schemes of Representative Noncovalent Protein−DNA Conjugation Methods[a]



[a](A) Biotin−monoavidin binding,[38] (B) NTA-Ni^{2+}-hexahistidine binding,[39] (C) ligand−protein binding (aptamer based),[45] (D) antigen−antibody binding,[47] (E) DNA-cofactor-apoenzyme reconstitution,[49] and (F) DNA binding protein (zinc finger protein).[55]

streptavidin (STV)−biotin interactions.[36,37] This is a convenient method because biotinylated oligonucleotides can be made by automated solid-phase synthesis and are commercially available, and STV−protein fusion can be obtained by protein engineering methods. Biotin−streptavidin binding also happens at mild conditions with very high affinity. The difficulty of controlling stoichiometry of DNA and protein can be overcome by an engineered monomeric avidin instead of the traditional tetrameric STV[38] (Scheme 1A).

Other ligand−protein binding methods that are commonly used include NTA-Ni^{2+}-Histag, aptamer−protein binding, and antigen−antibody binding, one of which, hexahistidine peptide (His_6), binds with nitrilotriacetic acid (NTA) ligand through nickel(II) ion[39] (Scheme 1B). Oligohistidine tags are commonly fused with proteins on N or C terminus using a nickel column for purification purposes.[40−42] One, two, or three NTA ligand-modified ssDNA are prepared with different dissociation constants (kD),[43] and kD decreased from 120 nM to 6 nM between bis- and trisNTA−DNA and His_6-tagged GFP. Another advantage of this method is the reversibility of ion induced binding, which can be released by chelators, such as ethylenediaminetetraacetate (EDTA). Alternatively, a recent study using cobalt(III) as mediator ion achieved inert binding.[44] More specific aptamer−protein binding has been popular mainly because of convenience that the ligand is formed by nucleic acid itself, so no chemical modification is needed (Scheme 1C). Binding affinity has been improved by assembling multivalent ligands with careful distance control on DNA nanostructures,[45] which has allowed this technique to be used to build spatially addressable multiprotein nanoarrays.[46] In addition to aptamer−protein binding, another method with high specificity and affinity is antigen−antibody binding[47] (Scheme 1D), which has been used to immobilize antibodies on a DNA scaffold. A 2D antibody array has been built with Fluorescene−IgG interaction.[48] By modifying two antibodies in close proximity to one antibody, uniform orientation and high density (~20 nm) nanoarrays were achieved, compared to the traditional solid-surface immobilization method.

b. Apoenzyme Reconstitution. For enzymes that have nondiffusible organic cofactors, an apoenzyme reconstitution method has been a convenient way to generate protein−DNA conjugates.[49] The principle is straightforward (Scheme 1E): (1) extract the cofactor from the active enzyme, leading to an inactive apoenzyme, (2) conjugate the cofactor with DNA, (3) reinsert DNA-cofactor into the apoenzyme, and achieve the active DNA-cofactor-enzyme conjugates. The enzyme activity is tuned with the DNA modified cofactor compared to wild-type cofactors. Among the cofactors, porphyrin and flavin derivatives

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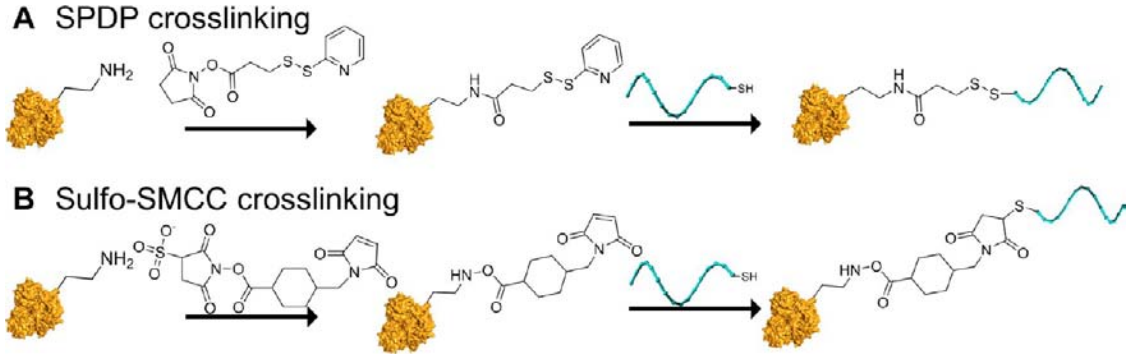
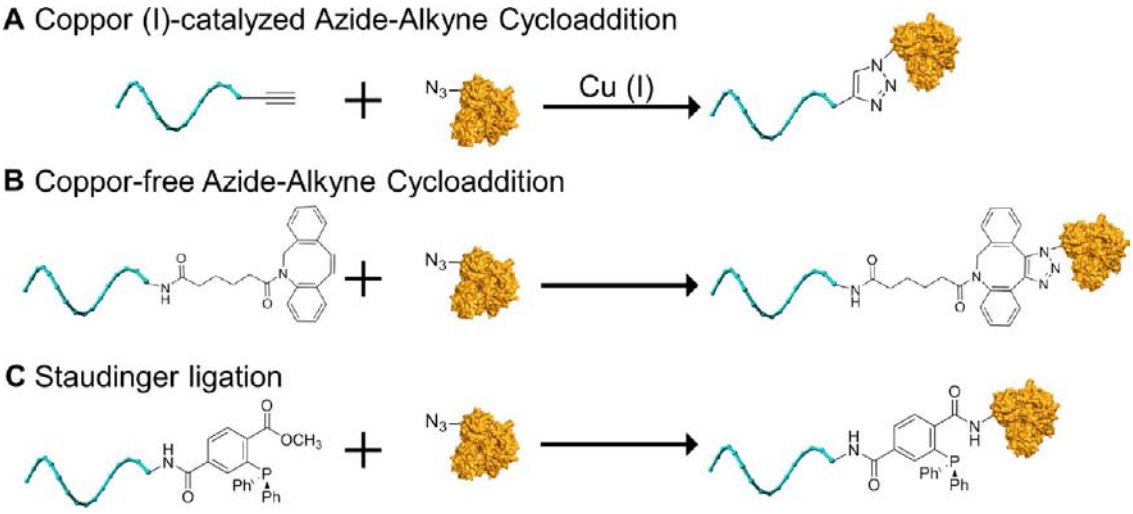
Scheme 2. Covalent Non-Site-Specific Protein−DNA Conjugation[a]



[a](A) *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP)[57] and (B) sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC)[58] cross-linkers. One end of the linker is covalently attached to the lysine side chains of protein and the other maleimide-functionalized end is subsequently coupled to thiolated DNA strands.

Scheme 3. Scheme of Site-Specific Azido-Protein-DNA Conjugation Chemistry[a]



[a]Including (A) Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction,[62] which reacts with terminal alkyne modified ssDNA; (B) Cu-free 1,3-dipolar cycloaddition reaction,[62] which reacts with dibenzocyclooctyne (DBCO)-modfied ssDNA; and (C) Staudinger ligation reaction,[63−65] which reacts with phosphine-modified ssDNA.

have been studied intensively, including heme-apo-myoglobin,[50,51] apo-HRP,[52] and flavin-apo-flavin reductase.[53] The unique activation and inactivation properties expand the conjugation method with switchable properties for target DNA detection[53] and tuning of enzyme activities.[54] The chemically modified cofactor may interfere with enzyme activity.

c. Domain Interactions: DNA-Binding Protein. Among a large number of DNA binding proteins, zinc-finger proteins (Zif) are one of the best characterized classes.[55] The zinc-finger protein has a DNA binding domain which binds a dsDNA region with specific sequences (∼10 bp) with nanomolar affinity[56] (Scheme 1F). This technique requires protein engineering to fuse the DNA binding domain with the enzyme of interest. With a variety of Zif that recognize different sequences, this method has been used to target multiple proteins to specific locations on DNA origami nanostructures.[56]

2.2. Covalent Non-Site-Specific Conjugation Method. Covalent protein−DNA conjugation methods are used to circumvent obstacles which result from dissociation of noncovalent, reversible interactions. A general method of wild-type protein and DNA conjugation is achieved by a heterobifunctional cross-linkers. For the most commonly used maleimide and *N*-hydroxysuccinimide (NHS) ester-derived cross-linkers, one end of the linker was covalently attached to the lysine side chains and the other maleimide-functionalized end was subsequently coupled to thiolated DNA strands[57] (Scheme 2). *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and sulfo-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC)[58] are commercially available cross-linkers, and the reaction procedures are mild and only take a few hours. This method only requires surface lysine residues and does not require protein engineering. Other than lysine, another cross-linker, dithiodipyridine, reacts with the surface cystine and further link to thio-modified ssDNA.[59] This not only saves time, but also avoids the problem caused by fusion, i.e., the protein may become insoluble after it is fused with tag. DNA can be modified on any protein that has lysine/cysteine on its surface. One problem with this method is that the stoichiometric control is limited. An average of DNA labeling stoichiometry can be controlled by titrating the amount of cross-linkers, and the reaction yield relies on the number of lysines exposed on the protein surface. An additional purification step is required to get the specific labeling ratio.

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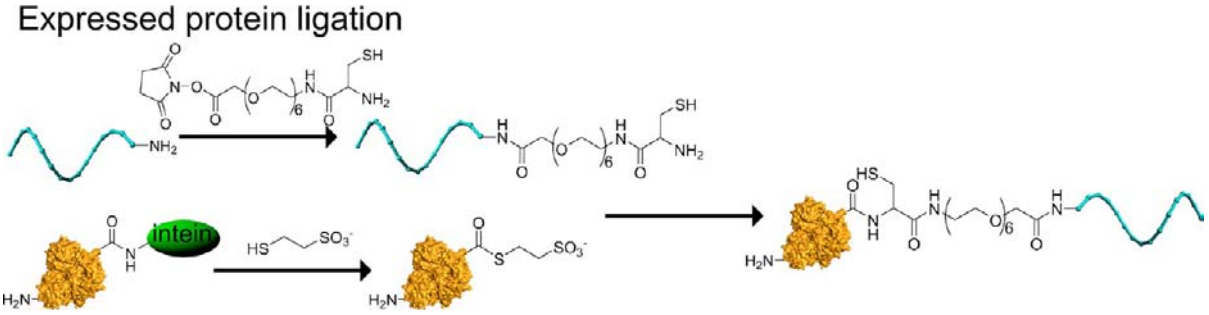
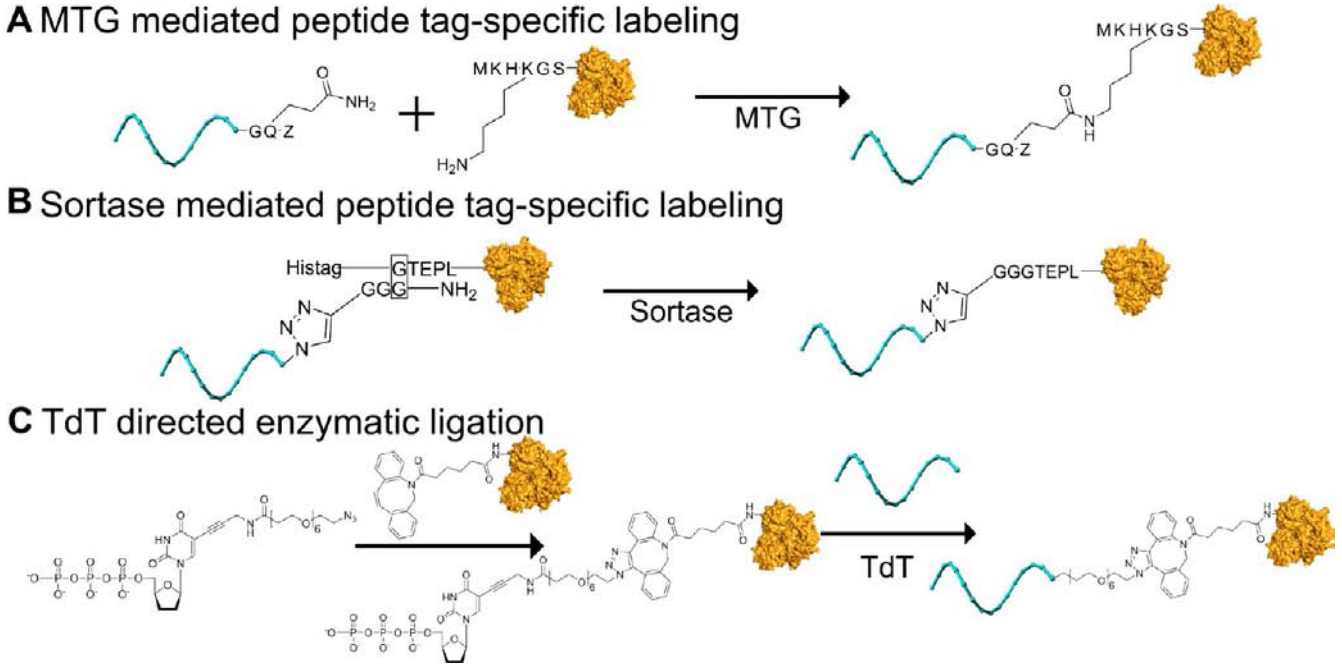
Scheme 4. Scheme of C-Terminal Fused Intein Protein−DNA Conjugation by Expressed Protein Ligation (EPL)[66a]



[a]The C-terminal intein fused protein reacts with mercaptoethansulfonic acid (MESNA) to generate thioester and then ligated to a cysteine-modified DNA.

Scheme 5. Scheme of Protein−DNA Ligation Mediated by Pure Enzyme System[a]



[a]Including (A) microbial transglutaminase (MTG),[68] (B) sortase,[69,70] and (C) terminal deoxynucleotidyl transferase (TdT).[72] (A) MTG catalyzes an acyl transfer reaction between *N*-carbobenzyloxyglutaminylglycine-DNA as the acyl-donor and a protein with short peptide tag containing the acyl-acceptor Lys residues (Met-Lys-His-Lys-Gly-Ser). (B) Sortase first recognizes a C-terminal −LPETG− sequence, and then transposes the N-terminal-GGG sequence with the glycine from the −LPETGGG− sequence, resulting in a peptide bond between protein and DNA. (C) TdT ligates the proteins that are activated by NTP through a copper-free DBCO-mediated click reaction to native DNA.

It is hard to control the exact conjugation site, which can cause difficulty if the lysine is close to the active site, and enzymatic activity can be highly reduced (or altered) after modification. The alteration of enzyme activity depends on the nature of the protein of interest and it is difficult to avoid this problem.

2.3. Covalent Site-Specific Conjugation Method. Site-specific DNA protein conjugation is typically achieved by expressing the protein of interest with a chemical handle that can subsequently react with functional groups on DNA at designed positions. These techniques require genetic manipulation to express the protein of interest with the desired mutations, which is challenging and has to be systematically optimized for any new proteins.

a. Azido−Protein DNA Conjugation. One commonly used site-specific protein−DNA ligation approach is based on azido-proteins. In this method, any protein of interest needs to be mutated with azide first. One popular method is to incorporate unnatural amino acids. A general strategy is to activate azido-homoalanine by the methionyl-tRNA synthetase (MetRS) of *Escherichia coli* and replace methionine in proteins expressed in methionine-depleted bacterial cultures.[60] Another way is using protein farnesyltransferase (PFTase) to label protein containing a C-terminal tetrapeptide tag with an azide-modified isoprenoid diphosphate.[61] After labeling the protein site-specifically with azide, several reactions can be done to further modify the protein with ssDNA (Scheme 3). The Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction which reacts with alkyne-modified ssDNA has been a classic method for site-specific labeling.[62] However, this technique has drawbacks, including (1) toxicity of Cu(I) to cells, (2) enzyme activity loss by Cu(I) binding to the active site, and (3) reduced reaction rate by Cu(I) disproportionation in an aqueous environment. Therefore, interest is growing in methods involving Cu-free 1,3-dipolar cycloaddition reactions and Staudinger ligation

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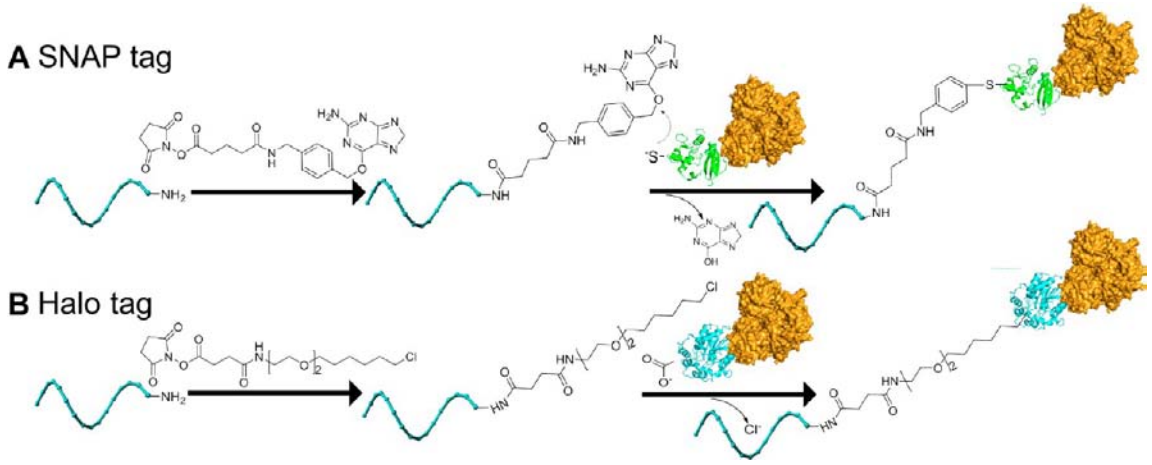
Scheme 6. Schemes of Protein−DNA Conjugation Using Self-Labeling Protein Tags[a]



[a]Including (A) human O6-alkylguanine-DNA-alkyltrans-ferase (SNAP) tagged protein-DNA conjugation[73,74] and (B) reaction of haloalkane dehalogenase (HaloTag) ligand-DNA and Halotagged-protein conjugation.[75] SNAP-tags irreversibly transfer the alkyl group from its substrate, O_6-benzylguanine-DNA, to one of its cysteine residues, creating a thioether covalent bond with the maleimide-DNA moiety. With the Halo-tag, nucleophilic displacement of the terminal chloride with Asp residue leads to a covalent alkyl-enzyme intermediate. O6-benzylguanine and 5-chlorohexane modified ssDNA can be prepared through amino-reactive *N*-hydroxysuccinimide (NHS) derivatives.[76]

reactions.[63] The Cu-free method involves reaction with a dibenzocyclooctyne (DBCO) functional groups.[62] Staudinger ligation involves reaction between azide and phosphine-modified components, and has been used to label ssDNA on azido-functionalized glycoproteins on cell surface.[64,65]

b. Expressed Protein Ligation (EPL). The expressed protein ligation method has been used to ligate both C-terminal[66] and N-terminal[67] intein-fused protein with peptide-modified ssDNA (Scheme 4). First, the target protein is fused to the construct of an intein, as well as an additional chitin binding domain (CBD) for the convenience of affinity purification using a chitin matrix. Then, a thioester of the target protein is achieved by reacting with mercaptoethansulfonic acid (MESNA). This protein can be ligated to a cysteine-modified DNA. While having the advantage of the well-defined stoichiometric composition and site-specific linkage, this method has challenges regarding insoluble intein fusions.

c. Enzymatic Ligation. High yield of protein−DNA conjugation can be achieved by enzymatic ligation. For example, microbial transglutaminase (MTG)[68] catalyzes an acyl transfer reaction between *N*-carbobenzyloxyglutaminylglycine (Z-QG) DNA and short peptide tag (Scheme 5A). The targeted proteins can be fused through genetic modification with a short peptide tag (Met-Lys-His-Lys-Gly-Ser) containing the acyl-acceptor Lys residues to N or C terminals. The oligonucleotides are modified with short peptide Z-QG, which functions as the acyl donor. This method has been applied to alkaline phosphatase and enhanced green fluorescent protein (eGFP) and has been used in DNA directed immobilization.[68]

Another method with a similar mechanism is the sortase-mediated protein−DNA conjugation.[69,70] The sortase enzyme catalyzes the formation of a covalent bond between two proteins by coupling two specific peptide sequences. Sortase first recognizes a C-terminal −LPETG− sequence, and then transposes the N-terminal-GGG sequence with the glycine from the −LPETGGG− sequence, resulting in a peptide bond between the two proteins (Scheme 5B). In detail, sortase cleaves the threonine−glycine bond via its active site cysteine residue and forms an acyl intermediate with threonine in the peptide, regenerating the active site cysteine on the sortase and conjugating the peptide-DNA to the N terminus of the protein. Note that at least one additional C-terminal amino acid is required for sortase to properly bind with the recognition sequence. Peptide−oligonucleotide conjugates can be prepared by automatic solid-phase synthesis.[71]

There is another option for replacing DNA modification by using terminal deoxynucleotidyl transferase (TdT), which can ligate native DNA to proteins coupled with nucleotide triphosphates.[72] TdT can accept nucleoside triphosphates tethered to large biomolecules as substrates and direct the ligation of the biomolecules to the 3′ end of any native oligodeoxynucleotides (Scheme 5C). The reaction is rapid and quantitative, while in mild and aqueous conditions. First, proteins are activated by NTP through a copper-free DBCO-mediated click reaction and then ligated to native DNA by TdT. The significant advantage of this method is the label-free ssDNA, allowing for batchwise functionalization of multiple staple strands that can be completed in one pot reaction. This leads to a high-throughput method for sensing purposes.

d. Self-Ligating Protein Tags. More recent site-specific protein−DNA conjugation techniques are based on the self-labeling protein tags which are fused by genetic modification to the targeted protein and catalyze the subsequent protein−DNA ligand reaction by themselves. The two popular examples are the human O6-alkylguanine-DNA-alkyltrans-ferase (hAGT, referred as "SNAP-tag"),[73,74] 20 kDa, and the haloalkane dehalogenase (referred as "HALO-tag"),[75,76] 34 kDa. The reaction procedures are similar except that different self-labeling tags have their own substrates, therefore different functional groups need to be modified on ssDNA targets accordingly (Scheme 6). SNAP-tags can irreversibly transfer the alkyl group from its substrate, O_6-benzylguanine-DNA, to one of its cysteine residues, creating a thioether covalent bond with the maleimide−DNA moiety. With the Halo-tag, nucleophilic displacement of the terminal chloride with Asp residue leads to a covalent alkyl-enzyme intermediate. O6-Benzylguanine and 5-chlorohexane modified ssDNA can be prepared through amino-reactive *N*-hydroxysuccinimide (NHS) derivatives.[76]

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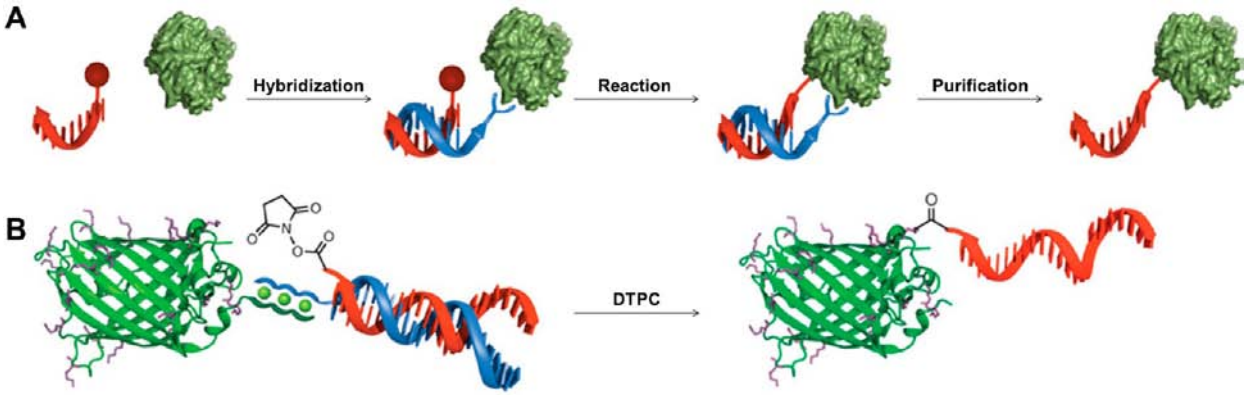
Scheme 7. Scheme and Characterization of DNA–Template Protein Conjugation (DTPC)[77a]



[a](A) General procedure of regioselective DNA-templated protein conjugation (DTPC) method. The guiding strand (blue waves) coordinates protein (green), and hybridize with the reacting strand (red waves), after the templated reaction of reacting strand and protein, the DNA–protein complex can be purified. (B) Detailed reaction scheme of the DTPC method: a Ni^{2+}-binding tris(NTA)-ssDNA (blue) is introduced to bind with His_6-tagged protein as a guide, which will hybridize with a second DNA strand (red) and subsequently cross-link with lysine that are only close to the metal-binding site. Images reproduced with permission: ref 77, copyright (2014) Nature publishing group.

The exact ratio of the ssDNA labeled on protein and the high yield of protein–DNA assembly can be achieved with mild protein reaction conditions. A considerable challenge for engineering artificial multienzyme systems in vivo is the stability of the protein–DNA complex. Compared to other protein tags, which form noncovalent linkage with DNA (i.e., His tag), the covalent linkage of protein–DNA appears to be much more stable in various buffers and potentially in blood circulation systems. Halo tag protein has also been used for in vivo cell labeling.[75] The high stability and specificity of this method make it a perfect candidate in therapeutic applications.

e. Template Directed Conjugation. To replace the complicated and problematic protein engineering process using protein tags, a novel and simpler way to create regioselective DNA–protein conjugates has been developed.[77] This DNA-templated protein conjugation (DTPC) method is a combination of metal-affinity probes and DNA-templated synthesis. The method relies on three components: a protein to be labeled that possesses an affinity for a metal ions, an ssDNA modified with a ligand functionality, and a complementary strand that carries an activated ester capable of reacting with the surface amino acids. As shown in Scheme 7, a metal-binding ssDNA is introduced to bind with His_6-tagged or wild-type metal-binding proteins as a guide, which will hybridize with a second DNA strand and subsequently cross-link with surface lysines which are only close to a metal-binding site. This DNA-templated protein conjugation method provides an alternative method for regiospecific DNA conjugation in contrast to technically challenging protein engineering; however, the site specificity has not yet been realized.

3. PROTEIN–DNA NANOSTRUCTURE ASSEMBLY AND PURIFICATION

3.1. Annealing. If a DNA hybridization method is used to attach proteins onto a DNA nanoscaffold, probes with 10 to 20 nt are normally used. An annealing process from 37 to 4 °C for 1–2 h is ideal with high hybridization yield and also maintains enzyme activity. Recently, both a single-molecule study[78] and bulk measurements[57,79,80] revealed that the number of anchoring points/attachment sites is crucial for the assembly yield and stability of the protein–DNA hybrid structure. With the assembly of small DNA tiles, it has been demonstrated that two attachment sites (single-stranded DNA probes), on both DNA structure and proteins, end up with high assembly yield (~90%), while one probe is lower (~50%).[57] For larger DNA nanostructures which have larger steric hindrance, i.e., rectangular DNA origami, four probes are incorporated in the structure to achieve high yield assembly.[79] Three probes have been used to capture a gold nanoparticle to achieve more than 96% yield on 24-helix bundle DNA origami.[80] This density-dependent dissociation and binding of hybridization kinetics has been revealed at the single molecule level using single-particle fluorescence resonance energy transfer (spFRET) as well.[78]

3.2. Purification. Since most of the protein–DNA conjugation methods lack stoichiometry control, an excess of protein or DNA is used to ensure high yield of the conjugation reaction. Nonspecific binding of ssDNA and protein increase the impurities, i.e., electrostatic interaction between positively charged proteins with negatively charged ssDNA. Purification of the product is crucial. The excess of reactants can be removed, and protein with the exact number of ssDNA labeling can be controlled. Due to the vast difference in charge between wild-type protein and ssDNA, ionic-exchange chromatography appears to be a suitable method. By increasing the salt concentration (i.e., NaCl), samples with different charges will be eluted out in the order of pure protein, protein labeled with 1 ssDNA, 2 ssDNA, and so on, until the peak of pure DNA. The eluted fractions can be characterized by gel electrophoresis.[55]

After the protein–DNA structure assembly, in order to remove the excess fold of free proteins, another round of purification steps is necessary. This is done by either the reversible affinity binding method[81] or size-exclusion chromatography.[57] In the affinity binding method, we can take advantage of the sequence addressable nature of DNA nanostructures, and different numbers of affinity molecules can be easily labeled on the structure. Depending on the different sizes and shapes of the DNA structure, the number and positions of the molecules need to be tested in order to maintain the structure and have a higher recovery yield. For size-exclusion chromatography, better purification can be

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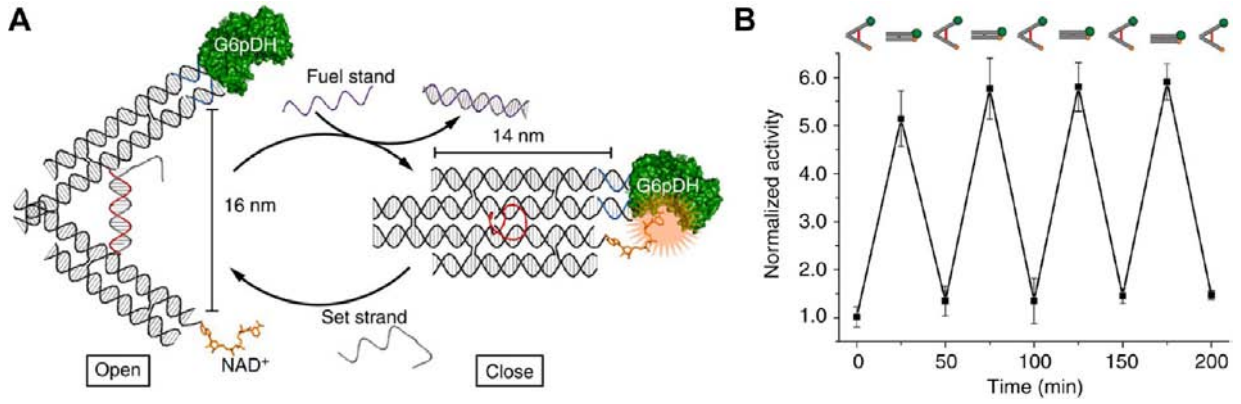

Figure 2. Enzyme-cofactor-DNA tweezer complex.[85] (A) Design of a tweezer complex using an enzyme-cofactor system including glucose-6-phosphate dehydrogenase (G6pDH) and nicotinamide adenine dinucleotide (NAD^+). The cofactor NAD^+ molecule is covalently linked to the end of one arm, while the enzyme is linked to the other. (B) Activity results showing that closed state revealed ~5-fold enhancement of enzyme activity compared to the open state; open and close states are reversible for at least 4 cycles. Images reproduced with permission: ref 85, copyright (2013) Nature publishing group.

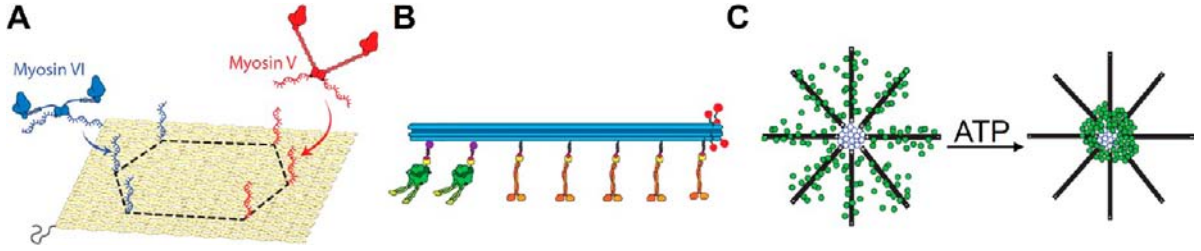

Figure 3. DNA nanostructure directed multiple motor proteins assembly. (A) Myosin lever arms with different stiffness organized on rectangular DNA origami that travel on a cellular actin network, Myosin V has a more rigid lever and VI has a more flexible lever.[95] (B) Two motor proteins with opposite polarity assembled on a 12-helix bundle DNA origami structure, showing a tug-of war behavior.[96] (C) Artificial self-organized transport system based on a microtubule motor protein−DNA complex that can assemble and disassemble with trigger molecules.[97] Images reproduced with permission: (A) ref 95, copyright (2014) Proceedings of the National Academy of Sciences; (B) ref 96, copyright (2012) American Association for the Advancement of Science; (C) ref 97, copyright (2013) Nature publishing group.

realized if the protein−DNA hybrid structure is much larger than the free protein; otherwise, it will be hard to achieve baseline separation.

4. APPLICATIONS

4.1. DNA Machine/Responsive Nanodevice. Other than simple switches based on dsDNA using modifications such as the photoisomerizable compounds,[82] more complex DNA nanostructure-based switchable nanodevices can be easily constructed and manipulated using DNA strand hybridization and displacement principles. DNA tweezers became a popular design in switching distances of two components. DNA tweezers can switch between open and closed states for at least four cycles.[83] Initially, the two arms of the tweezer are in a "closed" state, linked by a hairpin structure, and transferred to an "open" state by adding a fuel strand, which hybridizes with the hairpin loop and forms a rigid duplex. The switchability and kinetics are characterized by both gel electrophoresis and Förster resonance energy transfer (FRET). DNA tweezers can be used to actuate enzyme and enzyme cascades. One example is the use of DNA tweezers to control GOx/HRP cascade system. Due to the differences of open and closed state of two arms, the cascade activity (H_2O_2 production) differs by ~2-fold.[84] Another example is to use DNA tweezers to control the binding and dissociation of enzymes and its cofactors, as demonstrated by attaching glucose-6-phosphate dehydrogenase (G6pDH) and nicotinamide adenine dinucleotide (NAD^+)[85] to the two arms of the DNA tweezers (Figure 2). Since the enzyme is only active when directly "touching" the cofactor, closed state revealed ~5-fold enhancement of enzyme activity compared to the open state and the "on and off" state of enzyme activity can be reversibly regulated by adding fuel, and set DNA strands continuously.

4.2. Protein Patterning for Single Molecule and Structural Analysis. One of the advantages of DNA origami is that it serves as an excellent template for single molecule visualization.[86] A DNA nanoarray is different from traditional microarrays generated by the DNA-directed immobilization (DDI) method, with a higher order of spatial control. Because of this, DNA origami can be used as a molecular chip to display multiple probes for detection of various molecular interactions, i.e., RNA,[87] DNA,[88] distance-dependence of ligand-protein binding,[89] and chemical reactions on a single molecule level.[90−92] In order to address multiple components, orthogonal binding sites are needed. Both spatially addressable multiprotein nanoarrays[46,93] and protein nanoarrays with orientation control,[94] have been built.

4.3. DNA Scaffolded Motor Protein Networks. Researchers have studied motor protein behaviors by assembling multiple motor proteins on DNA scaffold. 2D rectangular DNA origami scaffold has been used to pattern a combination of myosin V with rigid lever and myosin VI with flexible level, and study the role of intermotor interactions on collective functions[95] (Figure 3A). Results of movement

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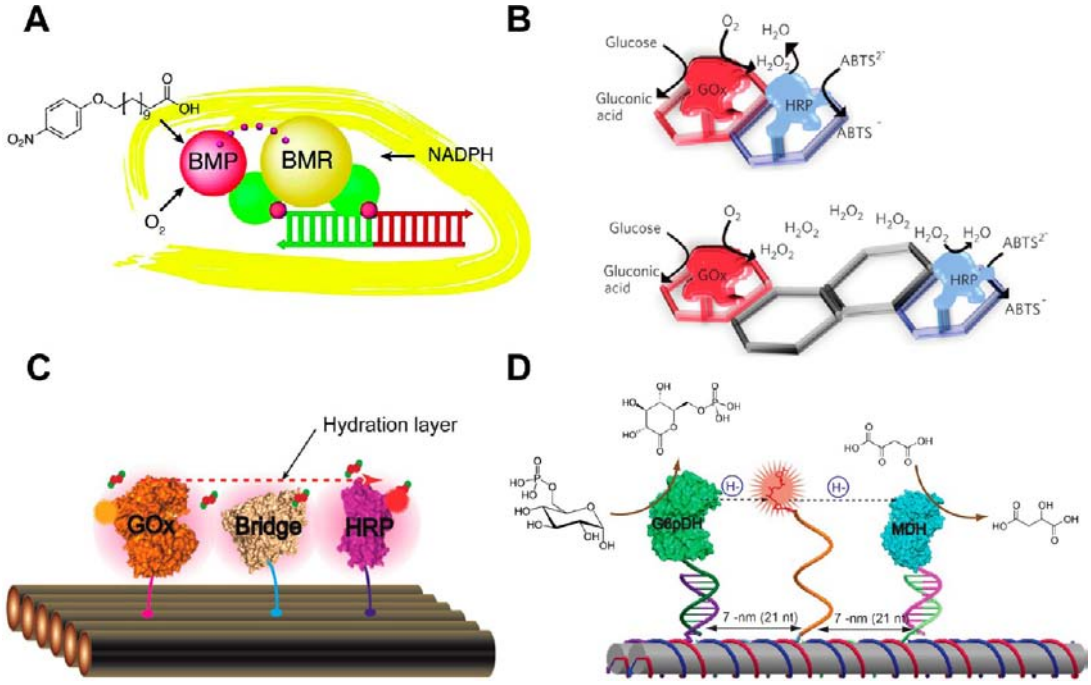

Figure 4. Artificial enzyme cascades organized by (A) double-stranded (ds) DNA,[98] (B) 2D tile based DNA arrays,[101] (C) rectangular DNA origami,[79] and (D) DNA tile.[57] (A) Design of distance-dependence study of cytochrome P450 BM3 with organizing the BMR reductase domain and the BMP porphyrin domain on dsDNA scaffold. (B) Scheme of glucose oxidase (GOx) and horseradish peroxidase (HRP) cascade organized with two different distances between GOx and HRP on two-hexagon (top) and four-hexagon strips (bottom). (C) Scheme of the assembled GOx/HRP pair with a protein bridge working as a connected hydration layer to facilitate H_2O_2 diffusion. (D) An artificial swinging-arm multienzyme complex consisting of glucose-6-phosphate dehydrogenase (G6pDH) and malic dehydrogenase (MDH) organized on a DNA DX tile, with a nicotinamide adenine dinucleotide (NAD^+)-modified ssDNA as the swinging arm, facilitating the transfer of hydrides. Images reproduced with permission: (A) ref 98, copyright (2011) American Chemical Society; (B) ref 101, copyright (2009) Nature publishing group; (C) ref 97, copyright (2012) American Chemical Society; (D) ref 57, copyright (2014) Nature publishing group.

trajectories on actin networks have shown that trajectory shape of multimotor scaffolds positively correlates with the stiffness of the myosin lever arm. Another study focused on kinesin and dynein motor proteins that transport cargo on microtubule tracks. Two motor proteins with opposite polarity are organized on 12-helix bundle DNA origami with different stoichiometry (Figure 3B). A "tug-of-war" behavior of the two proteins was observed.[96] An artificial self-organized transport system based on motor protein−DNA complex has been developed (Figure 3C). With trigger molecules, assembly and disassembly of the network, as well as loading and unloading of cargos on the track network can be realized.[97]

4.4. Engineering Enzymatic Pathways. In living systems, multienzymatic pathways are often physically and spatially organized onto scaffolds, clusters, and into microcompartments. By careful control of enzyme position, orientation, and ratio, the efficiency and specificity of enzymatic pathways in nature is extremely high. Spatial organization helps substrates flow between interacting proteins, and increases yield of sequential metabolic reactions. By exploiting the programmability of DNA nanostructures, key parameters including position, stoichiometry, and interenzyme distance can be manipulated and tested for optimal activity.

In order to fully understand and further engineer enzymatic pathways with maximum efficiency and limited cross-talk between signaling pathways, artificial multienzyme complexes have been built to mimic intracellular biocatalytic processes. An artificial enzyme cascade formed by glucose oxidase (GOx) and horseradish peroxidases (HRP) has been studied extensively as a model system. An advantage of the GOx/HRP cascade as a model system is the convenience of the enzyme cascade assay. In this case, the enzyme activity can be converted to optical signals with high sensitivity for detection.[79] In this Review, studies of GOx and HRP using double-stranded DNA (dsDNA), 1D, 2D, and origami as scaffolds are briefly summarized.

a. 1D DNA Scaffold. dsDNA has been used to bring two enzymes together to study the distance dependency[98,99] (Figure 4A). A 1D DNA nanowire[100,101] has been developed to attach tandem repeat units of GOx/HRP cascade, with the micrometer-long ssDNA working as the wire synthesized by rolling-circle amplification (RCA) process. The probes will hybridize with the enzyme-conjugated complementary strand. The activation of an enzyme cascade by the spatial positioning of the two enzymes (GOx and HRP) on the DNA template has been observed by activity assay monitoring $ABTS^-$ formation.

b. 2D DNA Structure Scaffold. Arranging multienzyme cascades on 2D complex geometric patterns was first studied by Willner.[102] In this work, the GOx-HRP cascade was organized with a 2D hexagonal DNA array (Figure 4B). Two distances between GOx and HRP were designed by varying the probe strand positions. Enzymes organized on the two-hexagon strips (shorter distances) gives higher activity than the four-hexagon strips. With shorter distances, intermediate (H_2O_2) diffusion has higher efficiency, which therefore results in higher cascade efficiency.

c. DNA Origami Scaffold. More accurate distance control is realized using DNA origami as a scaffold.[79] Instead of only comparing the assembled system with the free system, distances varying from 10, 20, 40, and 50 nm were systematically studied.

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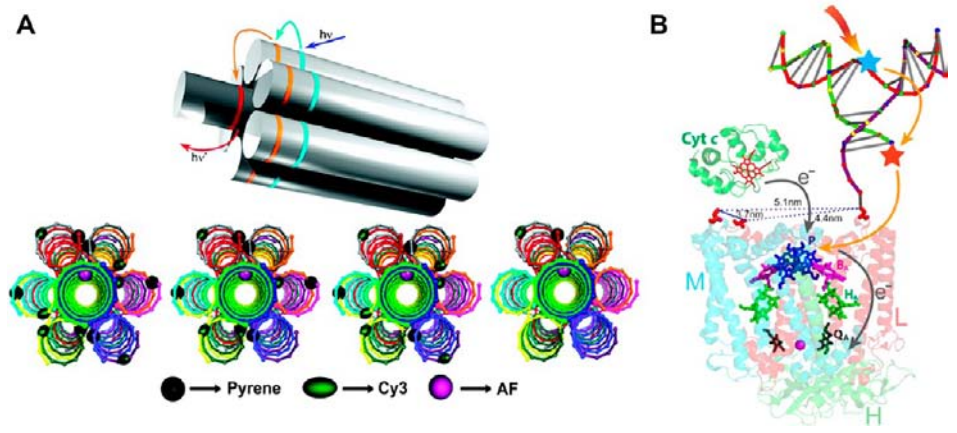

Figure 5. Artificial light-harvesting systems based on DNA nanostructures. (A) Artificial light-harvesting antenna built with a 6-helix bundle DNA nanostructure.[107] (B) Scheme of a DNA-directed reaction center system with expending light absorbing range by assembly of a Y-shapped DNA–dye complex on reaction center.[108] Images reproduced with permission: (A) ref 107, copyright (2011) American Chemical Society; (B) ref 108, copyright (2014) American Chemical Society.

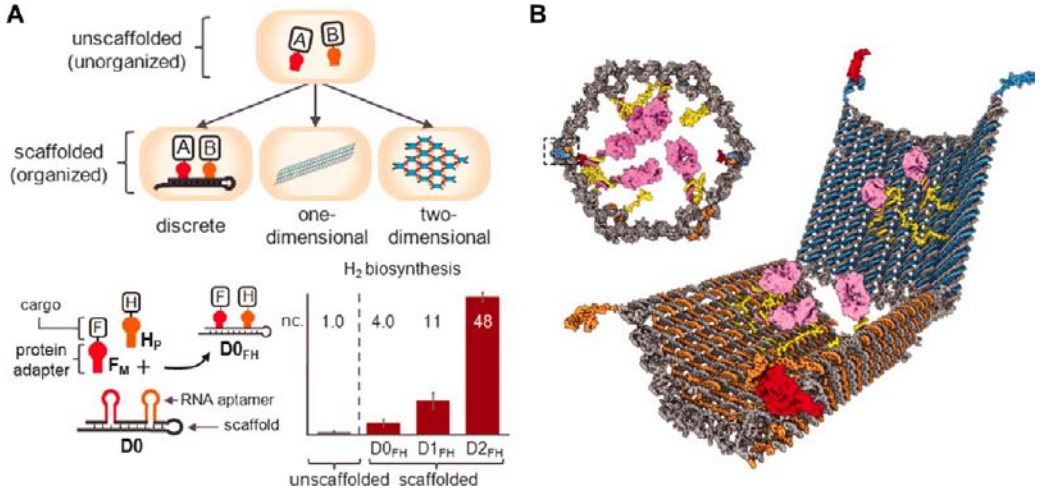

Figure 6. In vivo assembly of enzyme cascade by nucleic acid nanoscaffold. (A) Top: Scheme of two cascade proteins A and B scaffolded on discrete 1D and 2D self-assembled RNA scaffold.[109] Bottom left: Design of hydrogen-producing biosynthetic pathways with ferredoxin (F) and hydrogenase (H) enzyme cascade organized with RNA aptamer domains as probe. Bottom right: The scaffolding effects shown with hydrogen production enhancement after discrete 1D and 2D organization of two enzymes. (B) Scheme of aptamer-gated DNA nanorobot loaded with a protein payload.[110] Two DNA–aptamer locks are used to control opening and closing of the device. Images reproduced with permission: (A) ref 109, copyright (2011) American Association for the Advancement of Science; (B) ref 110, copyright (2012) American Association for the Advancement of Science.

Surprisingly, instead of a gradual decrease in cascade activity while increasing the interenzyme distances, a sharp transition was observed at 10 nm interenzyme distance, which was explained by hydration shell formation of the contacting proteins to facilitate the diffusion of H_2O_2 intermediate (Figure 4C). This mechanism is verified by constructing different sizes of protein bridges between GOx and HRP. The bridging protein changed the Brownian diffusion into a dimensionally limited diffusion through the hydration layer of the contacted enzymes. However, other than this specific GOx–HRP enzyme pair, more research is required for polar molecules other than H_2O_2 to fully understand the distance dependence of enzyme cascades. Recently, DNA origami nanostructures are also used to encapsulate GOx and HRP enzyme cascades into a confined environment with enhanced enzyme activity, although the mechanism of the caging effect remains to be further explored.[103]

d. Directed Substrate Channeling with Swinging Arms. More complex enzymatic pathways have been explored by adding a swinging arm linked cofactor between two enzymes[57] to understand substrate channeling. Substrate channeling[104] is used by nature for direct transfer of intermediate from one enzyme to a proximal enzyme to facilitate cascade activity. In this work, a multienzyme complex has been created based on DNA scaffolding, with an artificial swinging arm positioned between two coupled dehydrogenases, glucose-6-phosphate dehydrogenase (G6pDH) and malic dehydrogenase (MDH) (Figure 4D). This arm can swing between two enzymes via a flexible linker (20T single-strand), allowing the direct transfer of substrate molecules between multiple active sites within the complex. By employing a DNA scaffold, precise control over the spatial parameters of the individual components within the assembled complex is realized. The swinging behavior of the covalently linked "arm" is verified by single-molecule FRET experiment. Bulk activity results showed that the channeled

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NAD−protein complex has ∼90-fold enhancement activity compared to free, and by changing the relative ratio of two enzymes, activity can be further increased.

4.5. Photosynthetic Complexes/Light Harvesting Networks. People have been studying light harvesting systems for decades,[105] and DNA nanostructures shed light on creating artificial photosynthetic complexes in order to understand the mechanism and modulate the energy transfer efficiency.[105,106] In work using DNA nanostructures to study photosynthetic systems, an artificial light-harvesting antenna has been constructed by assembling donor−acceptors in a ring-like structure on a 6-helix-bundle DNA tile[107] (Figure 5A). More recently, an artificial photosynthetic complex has been created by site-specifically modifying a Y-shaped DNA nanostructure with dyes for stepwise energy transfer to a reaction center protein[108] (Figure 5B). Experimental results showed that Cytochrome c oxidation (monitored at 550 nm) of DNA-dye-RC complex has dramatically increased in comparison to wild-type RC with the excitation of Cy5 dye. The results indicate that the spectrum of the reaction center can be tuned and optimized by DNA directed artificial light harvesting systems.

4.6. In Vivo Regulation and Sensing. DNA nanostructures as scaffolds to organize multienzyme pathways have largely remained limited to in vitro applications. In contrast, RNA can be produced via the transcription machinery and forms stable interactions in vivo.[109−112] The first breakthrough of in vivo cascading of two enzymes was realized using a self-assembled RNA scaffold inside cells.[109] In this case, rationally designed RNA isothermal assemblies were successfully applied to form functionally discrete 1D and 2D scaffolds in vivo. These RNA scaffolds were functionalized by capturing proteins with RNA aptamer domains (Figure 6A). The scaffolding effects led to improved hydrogen production after organization of hydrogen-producing biosynthetic pathways on RNA nanoscaffolds. At this stage, RNA nanostructures are still limited and only a handful rationally designed RNA 2D and 3D structures have been reported.[112]

Another approach for in vivo regulation is assembling the structure in vitro and delivering the complex onto or into targeted cells. One successful example of this is the targeted delivery of a DNA nanobox for controlled drug release (in this case an antibody) with the "key" molecules displayed on cell surface and the interactive "lock" labeled on the DNA nanobox[110,113,114] (Figure 6B).

5. CONCLUSION AND FUTURE PERSPECTIVES

This Review has discussed different ways to use DNA nanostructures as scaffolds to organize biomolecules with programmability. A few technical challenges need to be met to realize better control and creation of DNA scaffolded biomolecular pathways and interactive networks, which are briefly identified below.

5.1. Method: Site-Specific Bioconjugation. A higher order of orientation and spatial control of proteins on a DNA scaffold is a must for true biomimicry. In order to achieve this, a more general site-specific protein−DNA conjugation that does not rely on protein engineering processes needs to be developed. One promising method is the N-terminal site-specific chemical modification method. Francis's group has developed N-terminal transamination chemistry to site-specifically modify proteins with fluorophores and PEG.[115−117] Due to the convenience of DNA modification with a variety of functional groups, DNA−protein conjugation methods developed from N-terminal click chemistry might become a viable approach to program site-specific conjugation.

5.2. Regulation: Orientation Control. With a convenient site-specific protein−DNA conjugation approach in hand, orientation control of the enzyme active sites could potentially be realized. It will be interesting to study enzyme cascades with the active sites facing each other, compared to the same distances with active sites facing away from each other (Figure 7). A higher level of angstrom-scale distance control may be achieved by constructing RNA, DNA, and protein hybrid structures as scaffolds, but these need better understanding of the design space.

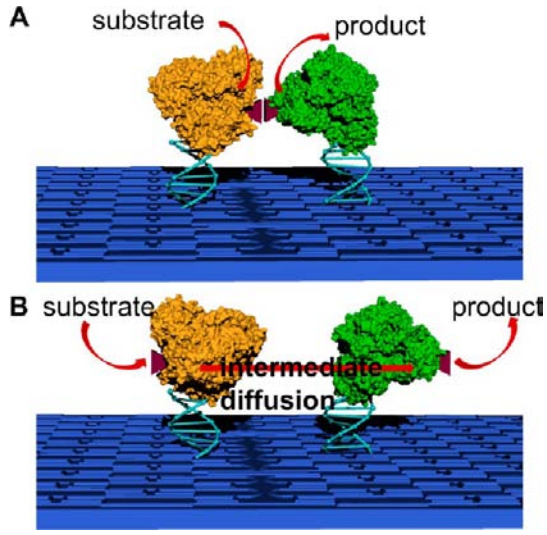

Figure 7. Schematics illustrating how orientation of enzymes on scaffold affects the enzyme cascade efficiency. With the same anchoring positions of DNA probes on DNA scaffold, when the two active sites of enzymes are facing each other (A), minimum intermediate diffusion is required which may results in higher cascade efficiency, while intermediate diffusion is required if the active sites face opposite with each other (B), which may decrease the substrate diffusion efficiency.

5.3. Organization: Organizing 2D and 3D Protein Network Using Designer DNA Crystals. Arranging proteins in a 2D network has been achieved using self-assembled 2D DNA tile systems. Extending this into rationally designed 3D networks remains to be explored. Since the first rationally designed, self-assembling DNA crystal structure was achieved,[14] researchers have started to functionalize the crystal with fluorophores.[118] Using DNA crystals to organize protein is one way to fix protein orientation by the nature of crystallization (Figure 8). It will be interesting to study how these 3D networks affect the enzyme pathway efficiency compared to the single-particle system.

5.4. Function: Protein (Antibody) Modified DNA Nanostructure for Cell Targeting. The mechanism of cellular uptake of DNA nanostructures remains mysterious. Is it shape-dependent, size-dependent, or charge-dependent? From recent studies, DNA tetrahedron nanostructures were found to enter only cytoplasm[119] and rectangularly shaped DNA origami can enter both cytoplasm and other acidic organelles.[120] In contrast, mechanisms of protein cell targeting by antibody−receptor binding are well studied. Combining

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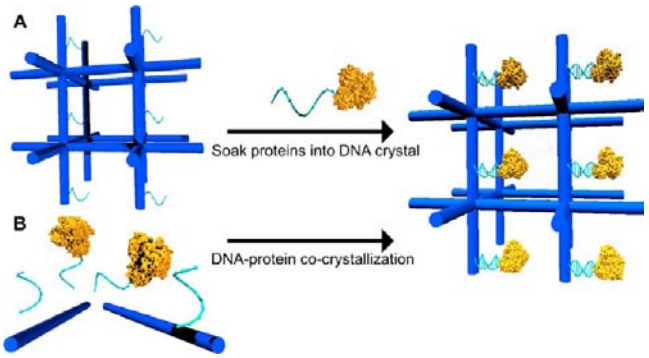

Figure 8. Schematics illustrating the organization of enzymes in 3D DNA crystals.

both properties by conjugating DNA−antibody complexes may lead us to reliable cell targeting for therapeutic purposes.

5.5. Function: In Vivo Enzymatic Pathway Engineering. It will be interesting to use DNA nanostructures to control the pathways to enable enzyme-catalyzed production of novel molecules and energy conversion in vivo. Still, challenges remain in the transfection efficiency of DNA nanostructures, enzyme−DNA complex stability, and in vivo assembly of enzymes and DNA nanostructures.

5.6. Nanorobots Integrating Functionality of Proteins and Programmability of DNA. Incorporating nucleic acid machinery into cells may lead to the design and construction of theranostic nanodevices for in vivo applications. It will be interesting to integrate nanosystems that combine molecular walkers, cargo, and tracks to achieve complex motions on 2D and 3D surfaces, and employ the scaffold to activate and deactivate a certain enzymatic pathway.

5.7. Concluding Remarks. The rapid developments in designing and constructing DNA nanostructures of high complexity has opened up a great opportunity to control the assembly of various biomolecules; yet, the integration between DNA and these biomolecules is still in its infancy. While experimental demonstrations using various DNA scaffolds to organize protein networks have led to functionalities that mimic nature's capabilities, theoretical modeling and systematic experiments are needed to gain a better understanding of the important parameters governing DNA directed biomolecular assembly. Indeed, DNA nanotechnology provides a highly programmable platform to realize these tasks in a controlled fashion.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hao.yan@asu.edu. Tel: (480) 727-8570. Fax: (480) 727-2378.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Seeman, N. C. (1982) Nucleic acid junctions and lattices. *J. Theor. Biol. 99*, 237−247.
(2) Seeman, N. C. (2003) DNA in a material world. *Nature 421*, 427−431.
(3) LaBean, T. H., Yan, H., Kopatsch, J., Liu, F., Winfree, E., Reif, J. H., and Seeman, N. C. (2000) Construction, analysis, ligation, and self-assembly of DNA triple crossover complexes. *J. Am. Chem. Soc. 122*, 1848−1860.
(4) Yan, H., Park, S. H., Finkelstein, G., Reif, J. H., and LaBean, T. H. (2003) DNA-templated self-assembly of protein arrays and highly conductive nanowires. *Science 301*, 1882−1884.
(5) He, Y., Ye, T., Su, M., Zhang, C., Ribbe, A. E., Jiang, W., and Mao, C. (2008) Hierarchical self-assembly of DNA into symmetric supramolecular polyhedra. *Nature 452*, 198−201.
(6) Lund, K., Liu, Y., Lindsay, S., and Yan, H. (2005) Self-assembling a molecular pegboard. *J. Am. Chem. Soc. 127*, 17606−17607.
(7) Liu, Y., Ke, Y., and Yan, H. (2005) Self-assembly of symmetric finite-size DNA nanoarrays. *J. Am. Chem. Soc. 127*, 17140−17141.
(8) He, Y., Chen, Y., Liu, H., Ribbe, A. E., and Mao, C. (2005) Self-assembly of hexagonal DNA two-dimensional (2D) arrays. *J. Am. Chem. Soc. 127*, 12202−12203.
(9) Mitchell, J. C., Harris, J. R., Malo, J., Bath, J., and Turberfield, A. J. (2004) Self-assembly of chiral DNA nanotubes. *J. Am. Chem. Soc. 126*, 16342−16343.
(10) Wilner, O. I., Orbach, R., Henning, A., Teller, C., Yehezkeli, O., Mertig, M., Harries, D., and Willner, I. (2011) Self-assembly of DNA nanotubes with controllable diameters. *Nat. Commun. 2*, 540.
(11) Goodman, R. P., Schaap, I. A. T., Tardin, C. F., Erben, C. M., Berry, R. M., Schmidt, C. F., and Turberfield, A. J. (2005) Rapid chiral assembly of rigid DNA building blocks for molecular nanofabrication. *Science 310*, 1661−1665.
(12) Aldaye, F. A., and Sleiman, H. F. (2007) Modular access to structurally switchable 3D discrete DNA assemblies. *J. Am. Chem. Soc. 129*, 13376−13377.
(13) Erben, C. M., Goodman, R. P., and Turberfield, A. J. (2007) A self-assembled DNA bipyramid. *J. Am. Chem. Soc. 129*, 6992−6993.
(14) Zheng, J., Birktoft, J. J., Chen, Y., Wang, T., Sha, R., Constantinou, P. E., Ginell, S. L., Mao, C., and Seeman, N. C. (2009) From molecular to macroscopic via the rational design of a self-assembled 3D DNA crystal. *Nature 461*, 74−77.
(15) Rothemund, P. W. K. (2006) Folding DNA to create nanoscale shapes and patterns. *Nature 440*, 297−302.
(16) Douglas, S. M., Marblestone, A. H., Teerapittayanon, S., Vazquez, A., Church, G. M., and Shih, W. M. (2009) Rapid prototyping of 3D DNA-origami shapes with caDNAno. *Nucleic Acids Res. 37*, 5001−5006.
(17) Andersen, E. S., Dong, M., Nielsen, M. M., Jahn, K., Subramani, R., Mamdouh, W., Golas, M. M., Sander, B., Stark, H., Oliveira, C. L. P., et al. (2009) Self-assembly of a nanoscale DNA box with a controllable lid. *Nature 459*, 73−76.
(18) Dietz, H., Douglas, S. M., and Shih, W. M. (2009) Folding DNA into twisted and curved nanoscale shapes. *Science 325*, 725−730.
(19) Douglas, S. M., Dietz, H., Liedl, T., Hogberg, B., Graf, F., and Shih, W. M. (2009) Self-assembly of DNA into nanoscale three-dimensional shapes. *Nature 459*, 414−418.
(20) Ke, Y., Douglas, S. M., Liu, M., Sharma, J., Cheng, A., Leung, A., Liu, Y., Shih, W. M., and Yan, H. (2009) Multilayer DNA origami packed on a square lattice. *J. Am. Chem. Soc. 131*, 15903−15908.
(21) Han, D., Pal, S., Nangreave, J., Deng, Z., Liu, Y., and Yan, H. (2011) DNA origami with complex curvatures in three-dimensional space. *Science 332*, 342−346.
(22) Han, D., Pal, S., Yang, Y., Jiang, S., Nangreave, J., Liu, Y., and Yan, H. (2013) DNA gridiron nanostructures based on four-arm junctions. *Science 339*, 1412−1415.
(23) Kato, T., Goodman, R. P., Erben, C. M., Turberfield, A. J., and Namba, K. (2009) High-resolution structural analysis of a DNA nanostructure by cryoEM. *Nano Lett. 9*, 2747−2750.
(24) Birkedal, V., Dong, M., Golas, M. M., Sander, B., Andersen, E. S., Gothelf, K. V., Besenbacher, F., and Kjems, J. (2011) Single molecule microscopy methods for the study of DNA origami structures. *Microsc. Res. Tech. 74*, 688−698.

DOI: 10.1021/acs.bioconjchem.5b00194
Bioconjugate Chem. 2015, 26, 1381−1395

(25) Bai, X.-c., Martin, T. G., Scheres, S. H., and Dietz, H. (2012) Cryo-EM structure of a 3D DNA-origami object. *Proc. Natl. Acad. Sci. U. S. A. 109*, 20012−20017.
(26) Opherden, L., Oertel, J., Barkleit, A., Fahmy, K., and Keller, A. (2014) Paramagnetic decoration of DNA origami nanostructures by Eu^{3+} coordination. *Langmuir 30*, 8152−8159.
(27) Pan, K., Kim, D.-N., Zhang, F., Adendorff, M. R., Yan, H., and Bathe, M. (2014) Lattice-free prediction of three-dimensional structure of programmed DNA assemblies. *Nat. Commun. 5*, 5578.
(28) Ke, Y., Ong, L. L., Shih, W. M., and Yin, P. (2012) Three-dimensional structures self-assembled from DNA bricks. *Science 338*, 1177−1183.
(29) Ke, Y., Ong, L. L., Sun, W., Song, J., Dong, M., Shih, W. M., and Yin, P. (2014) DNA brick crystals with prescribed depths. *Nat. Chem. 6*, 994−1002.
(30) Williams, B. A. R., Lund, K., Liu, Y., Yan, H., and Chaput, J. C. (2007) Self-assembled peptide nanoarrays: an approach to studying protein−protein interactions. *Angew. Chem. 119*, 3111−3114.
(31) Kuzyk, A., Laitinen, K. T., and Törmä, P. (2009) DNA origami as a nanoscale template for protein assembly. *Nanotechnology 20*, 235305.
(32) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjugate Chem. 1*, 165−187.
(33) Meyer, R., Giselbrecht, S., Rapp, B. E., Hirtz, M., and Niemeyer, C. M. (2014) Advances in DNA-directed immobilization. *Curr. Opin. Chem. Biol. 18*, 8−15.
(34) Stephanopoulos, N., and Francis, M. B. (2011) Choosing an effective protein bioconjugation strategy. *Nat. Chem. Biol. 7*, 876−884.
(35) Niemeyer, C. M. (2010) Semisynthetic DNA−protein conjugates for biosensing and nanofabrication. *Angew. Chem., Int. Ed. 49*, 1200−1216.
(36) Li, H., Park, S. H., Reif, J. H., LaBean, T. H., and Yan, H. (2003) DNA-templated self-assembly of protein and nanoparticle linear arrays. *J. Am. Chem. Soc. 126*, 418−419.
(37) Kuzuya, A., Kimura, M., Numajiri, K., Koshi, N., Ohnishi, T., Okada, F., and Komiyama, M. (2009) Precisely programmed and robust 2D streptavidin nanoarrays by using periodical nanometer-scale wells embedded in DNA origami assembly. *ChemBioChem 10*, 1811−1815.
(38) Howarth, M., Chinnapen, D. J., Gerrow, K., Dorrestein, P. C., Grandy, M. R., Kelleher, N. L., El-Husseini, A., and Ting, A. Y. (2006) A monovalent streptavidin with a single femtomolar biotin binding site. *Nat. Methods 3*, 267−273.
(39) Hochuli, E., Döbeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr. A 411*, 177−184.
(40) Shen, W., Zhong, H., Neff, D., and Norton, M. L. (2009) NTA directed protein nanopatterning on DNA origami nanoconstructs. *J. Am. Chem. Soc. 131*, 6660−6661.
(41) Shimada, J., Maruyama, T., Hosogi, T., Tominaga, J., Kamiya, N., and Goto, M. (2008) Conjugation of DNA with protein using His-tag chemistry and its application to the aptamer-based detection system. *Biotechnol. Lett. 30*, 2001−2006.
(42) Kapanidis, A. N., Ebright, Y. W., and Ebright, R. H. (2001) Site-specific incorporation of fluorescent probes into protein: hexahistidine-tag-mediated fluorescent labeling with (Ni^{2+}:nitrilotriacetic acid)$_n$−fluorochrome conjugates. *J. Am. Chem. Soc. 123*, 12123−12125.
(43) Goodman, R. P., Erben, C. M., Malo, J., Ho, W. M., McKee, M. L., Kapanidis, A. N., and Turberfield, A. J. (2009) A facile method for reversibly linking a recombinant protein to DNA. *ChemBioChem 10*, 1551−1557.
(44) Wegner, S. V., and Spatz, J. P. (2013) Cobalt(III) as a stable and inert mediator ion between NTA and His6-tagged proteins. *Angew. Chem., Int. Ed. 52*, 7593−7596.
(45) Williams, B. A. R., Diehnelt, C. W., Belcher, P., Greving, M., Woodbury, N. W., Johnston, S. A., and Chaput, J. C. (2009) Creating protein affinity reagents by combining peptide ligands on synthetic DNA scaffolds. *J. Am. Chem. Soc. 131*, 17233−17241.
(46) Chhabra, R., Sharma, J., Ke, Y., Liu, Y., Rinker, S., Lindsay, S., and Yan, H. (2007) Spatially addressable multiprotein nanoarrays templated by aptamer-tagged DNA nanoarchitectures. *J. Am. Chem. Soc. 129*, 10304−10305.
(47) Voss, E. W., and Lopatin, D. E. (1971) Fluorescein. Hapten and antibody active-site probe. *Biochemistry 10*, 208−213.
(48) He, Y., Tian, Y., Ribbe, A. E., and Mao, C. (2006) Antibody nanoarrays with a pitch of ∼20 nanometers. *J. Am. Chem. Soc. 128*, 12664−12665.
(49) Fruk, L., Kuo, C.-H., Torres, E., and Niemeyer, C. M. (2009) Apoenzyme reconstitution as a chemical tool for structural enzymology and biotechnology. *Angew. Chem., Int. Ed. 48*, 1550−1574.
(50) Fruk, L., and Niemeyer, C. M. (2005) Covalent Hemin−DNA adducts for generating a novel class of artificial heme enzymes. *Angew. Chem., Int. Ed. 44*, 2603−2606.
(51) Fruk, L., Müller, J., and Niemeyer, C. M. (2006) Kinetic analysis of semisynthetic peroxidase enzymes containing a covalent DNA−heme adduct as the cofactor. *Chem.—Eur. J. 12*, 7448−7457.
(52) Fruk, L., Müller, J., Weber, G., Narváez, A., Domínguez, E., and Niemeyer, C. M. (2007) DNA-directed immobilization of horseradish peroxidase−DNA conjugates on microelectrode arrays: towards electrochemical screening of enzyme libraries. *Chem.—Eur. J. 13*, 5223−5231.
(53) Simon, P., Dueymes, C., Fontecave, M., and Décout, J.-L. (2005) DNA detection through signal amplification by using NADH:flavin oxidoreductase and oligonucleotide−flavin conjugates as cofactors. *Angew. Chem., Int. Ed. 44*, 2764−2767.
(54) Gianneschi, N. C., and Ghadiri, M. R. (2007) Design of molecular logic devices based on a programmable DNA-regulated semisynthetic enzyme. *Angew. Chem. 119*, 4029−4032.
(55) Klug, A., and Schwabe, J. W. (1995) Protein motifs 5. Zinc fingers. *FASEB J. 9*, 597−604.
(56) JH, L., BM, L., and PE, W. (2001) Zinc finger proteins: new insights into structural and functional diversity. *Curr. Opin. Struct. Biol. 11*, 39.
(57) Fu, J., Yang, Y. R., Johnson-Buck, A., Liu, M., Liu, Y., Walter, N. G., Woodbury, N. W., and Yan, H. (2014) Multi-enzyme complexes on DNA scaffolds capable of substrate channelling with an artificial swinging arm. *Nat. Nano 9*, 531−536.
(58) Erben, C. M., Goodman, R. P., and Turberfield, A. J. (2006) Single-molecule protein encapsulation in a rigid DNA cage. *Angew. Chem. 118*, 7574−7577.
(59) Cecconi, C., Shank, E., Dahlquist, F., Marqusee, S., and Bustamante, C. (2008) Protein-DNA chimeras for single molecule mechanical folding studies with the optical tweezers. *Eur. Biophys J. 37*, 729−738.
(60) Kiick, K. L., Saxon, E., Tirrell, D. A., and Bertozzi, C. R. (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc. Natl. Acad. Sci. U. S. A. 99*, 19−24.
(61) Duckworth, B. P., Chen, Y., Wollack, J. W., Sham, Y., Mueller, J. D., Taton, T. A., and Distefano, M. D. (2007) A universal method for the preparation of covalent protein−DNA conjugates for use in creating protein nanostructures. *Angew. Chem. 119*, 8975−8978.
(62) ávan Berkel, S. S., Floris, P., ávan Hest, J. C., and ávan Delft, F. L. (2010) Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+ 2) cycloaddition. *Chem. Commun. 46*, 97−99.
(63) Köhn, M., and Breinbauer, R. (2004) The Staudinger ligation—a gift to chemical biology. *Angew. Chem., Int. Ed. 43*, 3106−3116.
(64) Chandra, R. A., Douglas, E. S., Mathies, R. A., Bertozzi, C. R., and Francis, M. B. (2006) Programmable cell adhesion encoded by DNA hybridization. *Angew. Chem. 118*, 910−915.
(65) Douglas, E. S., Chandra, R. A., Bertozzi, C. R., Mathies, R. A., and Francis, M. B. (2007) Self-assembled cellular microarrays patterned using DNA barcodes. *Lab Chip 7*, 1442−1448.
(66) Takeda, S., Tsukiji, S., and Nagamune, T. (2004) Site-specific conjugation of oligonucleotides to the C-terminus of recombinant

DOI: 10.1021/acs.bioconjchem.5b00194
Bioconjugate Chem. 2015, 26, 1381−1395

protein by expressed protein ligation. *Bioorg. Med. Chem. Lett. 14*, 2407−2410.
(67) Takeda, S., Tsukiji, S., Ueda, H., and Nagamune, T. (2008) Covalent split protein fragment-DNA hybrids generated through N-terminus-specific modification of proteins by oligonucleotides. *Org. Biomol. Chem. 6*, 2187−2194.
(68) Tominaga, J., Kemori, Y., Tanaka, Y., Maruyama, T., Kamiya, N., and Goto, M. (2007) An enzymatic method for site-specific labeling of recombinant proteins with oligonucleotides. *Chem. Commun.*, 401−403.
(69) Theile, C. S., Witte, M. D., Blom, A. E. M., Kundrat, L., Ploegh, H. L., and Guimaraes, C. P. (2013) Site-specific N-terminal labeling of proteins using sortase-mediated reactions. *Nat. Protoc. 8*, 1800−1807.
(70) Koussa, M. A., Sotomayor, M., and Wong, W. P. (2014) Protocol for sortase-mediated construction of DNA−protein hybrids and functional nanostructures. *Methods 67*, 134−141.
(71) Venkatesan, N., and Kim, B. H. (2006) Peptide conjugates of oligonucleotides: synthesis and applications. *Chem. Rev. 106*, 3712−3761.
(72) Sørensen, R. S., Okholm, A. H., Schaffert, D., Kodal, A. L. B., Gothelf, K. V., and Kjems, J. (2013) Enzymatic ligation of large biomolecules to DNA. *ACS Nano 7*, 8098−8104.
(73) Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003) A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol. 21*, 86−89.
(74) Jongsma, M. A., and Litjens, R. H. G. M. (2006) Self-assembling protein arrays on DNA chips by auto-labeling fusion proteins with a single DNA address. *Proteomics 6*, 2650−2655.
(75) Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., et al. (2008) HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol. 3*, 373−382.
(76) Saccà, B., Meyer, R., Erkelenz, M., Kiko, K., Arndt, A., Schroeder, H., Rabe, K. S., and Niemeyer, C. M. (2010) Orthogonal protein decoration of DNA origami. *Angew. Chem., Int. Ed. 49*, 9378−9383.
(77) Rosen, C. B., KodalAnne, L. B., Nielsen, J. S., Schaffert, D. H., Scavenius, C., Okholm, A. H., Voigt, N. V., Enghild, J. J., Kjems, J., Tørring, T., et al. (2014) Template-directed covalent conjugation of DNA to native antibodies, transferrin and other metal-binding proteins. *Nat. Chem. 6*, 804−809.
(78) Johnson-Buck, A., and Walter, N. G. (2014) Discovering anomalous hybridization kinetics on DNA nanostructures using single-molecule fluorescence microscopy. *Methods 67*, 177−184.
(79) Fu, J., Liu, M., Liu, Y., Woodbury, N. W., and Yan, H. (2012) Interenzyme substrate diffusion for an enzyme cascade organized on spatially addressable DNA nanostructures. *J. Am. Chem. Soc. 134*, 5516−5519.
(80) Kuzyk, A., Schreiber, R., Fan, Z., Pardatscher, G., Roller, E.-M., Hogele, A., Simmel, F. C., Govorov, A. O., and Liedl, T. (2012) DNA-based self-assembly of chiral plasmonic nanostructures with tailored optical response. *Nature 483*, 311−314.
(81) Numajiri, K., Yamazaki, T., Kimura, M., Kuzuya, A., and Komiyama, M. (2010) Discrete and active enzyme nanoarrays on DNA origami scaffolds purified by affinity tag separation. *J. Am. Chem. Soc. 132*, 9937−9939.
(82) You, M., Wang, R.-W., Zhang, X., Chen, Y., Wang, K., Peng, L., and Tan, W. (2011) Photon-regulated DNA-enzymatic nanostructures by molecular assembly. *ACS Nano 5*, 10090−10095.
(83) Zhou, C., Yang, Z., and Liu, D. (2012) Reversible regulation of protein binding affinity by a DNA machine. *J. Am. Chem. Soc. 134*, 1416−1418.
(84) Xin, L., Zhou, C., Yang, Z., and Liu, D. (2013) Regulation of an enzyme cascade reaction by a DNA machine. *Small 9*, 3088−3091.
(85) Liu, M., Fu, J., Hejesen, C., Yang, Y., Woodbury, N. W., Gothelf, K., Liu, Y., and Yan, H. (2013) A DNA tweezer-actuated enzyme nanoreactor. *Nat. Commun. 4*.
(86) Rajendran, A., Endo, M., and Sugiyama, H. (2012) Single-molecule analysis using DNA origami. *Angew. Chem., Int. Ed. 51*, 874−890.
(87) Ke, Y., Lindsay, S., Chang, Y., Liu, Y., and Yan, H. (2008) Self-assembled water-soluble nucleic acid probe tiles for label-free RNA hybridization assays. *Science 319*, 180−183.
(88) Subramanian, H. K. K., Chakraborty, B., Sha, R., and Seeman, N. C. (2011) The label-free unambiguous detection and symbolic display of single nucleotide polymorphisms on DNA origami. *Nano Lett. 11*, 910−913.
(89) Eberhard, H., Diezmann, F., and Seitz, O. (2011) DNA as a molecular ruler: interrogation of a tandem SH2 domain with self-assembled, bivalent DNA−peptide complexes. *Angew. Chem. 123*, 4232−4236.
(90) Tørring, T., Helmig, S., Ogilby, P. R., and Gothelf, K. V. (2014) Singlet oxygen in DNA nanotechnology. *Acc. Chem. Res. 47*, 1799−1806.
(91) Keller, A., Bald, I., Rotaru, A., Cauët, E., Gothelf, K. V., and Besenbacher, F. (2012) Probing electron-induced bond cleavage at the single-molecule level using DNA origami templates. *ACS Nano 6*, 4392−4399.
(92) Voigt, N. V., Torring, T., Rotaru, A., Jacobsen, M. F., Ravnsbaek, J. B., Subramani, R., Mamdouh, W., Kjems, J., Mokhir, A., Besenbacher, F., et al. (2010) Single-molecule chemical reactions on DNA origami. *Nat. Nano 5*, 200−203.
(93) Cohen, J. D., Sadowski, J. P., and Dervan, P. B. (2007) Programming multiple protein patterns on a single DNA nanostructure. *J. Am. Chem. Soc. 130*, 402−403.
(94) Kuzuya, A., Numajiri, K., and Komiyama, M. (2008) Accommodation of a single protein guest in nanometer-scale wells embedded in a "DNA Nanotape". *Angew. Chem., Int. Ed. 47*, 3400−3402.
(95) Hariadi, R. F., Cale, M., and Sivaramakrishnan, S. (2014) Myosin lever arm directs collective motion on cellular actin network. *Proc. Natl. Acad. Sci. U. S. A. 111*, 4091−4096.
(96) Derr, N. D., Goodman, B. S., Jungmann, R., Leschziner, A. E., Shih, W. M., and Reck-Peterson, S. L. (2012) Tug-of-war in motor protein ensembles revealed with a programmable DNA origami scaffold. *Science 338*, 662−665.
(97) Wollman, A. J. M., Sanchez-Cano, C., Carstairs, H. M. J., Cross, R. A., and Turberfield, A. J. (2014) Transport and self-organization across different length scales powered by motor proteins and programmed by DNA. *Nat. Nano 9*, 44−47.
(98) Erkelenz, M., Kuo, C.-H., and Niemeyer, C. M. (2011) DNA-mediated assembly of cytochrome P450 BM3 subdomains. *J. Am. Chem. Soc. 133*, 16111−16118.
(99) Niemeyer, C. M., Koehler, J., and Wuerdemann, C. (2002) DNA-directed assembly of bienzymic complexes from in vivo biotinylated NAD(P)H:FMN oxidoreductase and luciferase. *ChemBioChem 3*, 242−245.
(100) Wang, Z.-G., Wilner, O. I., and Willner, I. (2009) Self-assembly of aptamer−circular DNA nanostructures for controlled biocatalysis. *Nano Lett. 9*, 4098−4102.
(101) Wilner, O. I., Shimron, S., Weizmann, Y., Wang, Z.-G., and Willner, I. (2009) Self-assembly of enzymes on DNA scaffolds: en route to biocatalytic cascades and the synthesis of metallic nanowires. *Nano Lett. 9*, 2040−2043.
(102) Wilner, O. I., Weizmann, Y., Gill, R., Lioubashevski, O., Freeman, R., and Willner, I. (2009) Enzyme cascades activated on topologically programmed DNA scaffolds. *Nat. Nano 4*, 249−254.
(103) Fu, Y., Zeng, D., Chao, J., Jin, Y., Zhang, Z., Liu, H., Li, D., Ma, H., Huang, Q., Gothelf, K. V., et al. (2013) Single-step rapid assembly of DNA origami nanostructures for addressable nanoscale bioreactors. *J. Am. Chem. Soc. 135*, 696−702.
(104) Miles, E. W., Rhee, S., and Davies, D. R. (1999) The molecular basis of substrate channeling. *J. Biol. Chem. 274*, 12193−12196.
(105) Frischmann, P. D., Mahata, K., and Würthner, F. (2013) Powering the future of molecular artificial photosynthesis with light-

DOI: 10.1021/acs.bioconjchem.5b00194
Bioconjugate Chem. 2015, 26, 1381−1395

harvesting metallosupramolecular dye assemblies. *Chem. Soc. Rev. 42*, 1847−1870.
(106) Pullerits, T., and Sundström, V. (1996) Photosynthetic light-harvesting pigment-protein complexes: toward understanding how and why. *Acc. Chem. Res. 29*, 381−389.
(107) Dutta, P. K., Varghese, R., Nangreave, J., Lin, S., Yan, H., and Liu, Y. (2011) DNA-directed artificial light-harvesting antenna. *J. Am. Chem. Soc. 133*, 11985−11993.
(108) Dutta, P. K., Levenberg, S., Loskutov, A., Jun, D., Saer, R., Beatty, J. T., Lin, S., Liu, Y., Woodbury, N. W., and Yan, H. (2014) A DNA-directed light-harvesting/reaction center system. *J. Am. Chem. Soc. 136*, 16618−16625.
(109) Delebecque, C. J., Lindner, A. B., Silver, P. A., and Aldaye, F. A. (2011) Organization of intracellular reactions with rationally designed RNA assemblies. *Science 333*, 470−474.
(110) Douglas, S. M., Bachelet, I., and Church, G. M. (2012) A logic-gated nanorobot for targeted transport of molecular payloads. *Science 335*, 831−834.
(111) Delebecque, C. J., Silver, P. A., and Lindner, A. B. (2012) Designing and using RNA scaffolds to assemble proteins in vivo. *Nat. Protocols 7*, 1797−1807.
(112) Guo, P. (2010) The emerging field of RNA nanotechnology. *Nat. Nano 5*, 833−842.
(113) Fu, J., and Yan, H. (2012) Controlled drug release by a nanorobot. *Nat. Biotechnol. 30*, 407−408.
(114) Elbaz, J., and Willner, I. (2012) DNA origami: Nanorobots grab cellular control. *Nat. Mater. 11*, 276−277.
(115) Carrico, Z. M., Farkas, M. E., Zhou, Y., Hsiao, S. C., Marks, J. D., Chokhawala, H., Clark, D. S., and Francis, M. B. (2012) N-terminal labeling of filamentous phage to create cancer marker imaging agents. *ACS Nano 6*, 6675−6680.
(116) Palla, K. S., Witus, L. S., Mackenzie, K. J., Netirojjanakul, C., and Francis, M. B. (2014) Optimization and expansion of a site-selective N-methylpyridinium-4-carboxaldehyde-mediated transamination for bacterially expressed proteins. *J. Am. Chem. Soc. 137*, 1123−1129.
(117) Witus, L. S., Netirojjanakul, C., Palla, K. S., Muehl, E. M., Weng, C.-H., Iavarone, A. T., and Francis, M. B. (2013) Site-specific protein transamination using N-methylpyridinium-4-carboxaldehyde. *J. Am. Chem. Soc. 135*, 17223−17229.
(118) Rusling, D. A., Chandrasekaran, A. R., Ohayon, Y. P., Brown, T., Fox, K. R., Sha, R., Mao, C., and Seeman, N. C. (2014) Functionalizing designer DNA crystals with a triple-helical veneer. *Angew. Chem., Int. Ed. 53*, 3979−3982.
(119) Walsh, A. S., Yin, H., Erben, C. M., Wood, M. J. A., and Turberfield, A. J. (2011) DNA cage delivery to mammalian cells. *ACS Nano 5*, 5427−5432.
(120) Jiang, Q., Song, C., Nangreave, J., Liu, X., Lin, L., Qiu, D., Wang, Z.-G., Zou, G., Liang, X., Yan, H., et al. (2012) DNA origami as a carrier for circumvention of drug resistance. *J. Am. Chem. Soc. 134*, 13396−13403.

DOI: 10.1021/acs.bioconjchem.5b00194
Bioconjugate Chem. 2015, 26, 1381−1395