

Nucleic Acid Based Nanoreactors—Toward the Study of Multienzymatic Pathways

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Biological systems have used millions of years of evolution to produce enzymes that act as incredibly specific and efficient catalysts for important biochemical processes. Despite considerable effort into the use of enzymes for catalysis of industrial chemical reactions, the ability to successfully replicate the multi-enzymatic pathways observed in nature has been elusive. In this review, we focus on highlighting the recent work done utilizing the relatively new field of DNA nanotechnology to replicate multi-enzyme systems in the laboratory. By using canonical Watson-Crick base pairing rules, complex 1D, 2D, and 3D structures can be self-assembled from carefully designed DNA sequences with unprecedented control over the global addressability and molecular resolution of the system. We briefly cover techniques used to generate DNA-enzyme conjugates before discussing promising results in using DNA nanotechnology to assemble rationally designed multi-enzyme systems.

Keywords: DNA Nanotechnology, Enzyme Cascade, Protein Assembly.

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1. INTRODUCTION

Complex enzymatic pathways with numerous multistep reactions that possess high yields and specificities are very crucial for living systems.^{1–7} The key to the success of such multistep reactions is the relative orientation and distance of the enzymes, which allow for efficient substrate channeling between the active sites of enzymes in the complex.^{7–17} The use of efficient substrate channeling helps prevent loss of intermediates by diffusion, protects reactive intermediates from competing reactions (i.e., hydrolysis), increases the reaction flux, and reduces transient time scales.^{3, 18} In some natural metabolic systems, the efficient performance is achieved by organization of multiple enzymes into complexes having enzyme-to-enzyme channeling. For example, the crystal structure of

two different enzymes, tryptophan synthase and carbamoyl phosphate synthetase, reveal tunnels that connect catalytic sites within the enzymes.^{19, 20} A clear understanding of the benefits of such enzymes have lead scientists to develop biocatalysis techniques, which is a promising path towards environmentally benign methodologies for chemical manufacturing. These systems utilize biodegradable catalysts and mild reaction conditions, while still displaying high activity and selectivity.^{21, 22} However, the large-scale use of biocatalysis is often hampered due to limitations in the reusability and long-term stability of the enzymes.^{23, 24} Numerous efforts have been devoted by scientists around the world to overcome such drawbacks; one of these approaches is immobilization of enzyme.^{24–26} Immobilized enzymes can be more easily separated from the final product, can be recovered more efficiently, and possess higher stability, all of which improve the reusability of the catalyst. In general, enzymes can be immobilized in three different methods; binding to a carrier, encapsulation and cross-linking.^{21, 27–30} Synthetic resins, biopolymers and inorganic polymers are all used as carriers to support the immobilized enzyme. Encapsulation involves the synthesis of polymer material in the presence of the enzymes to be immobilized, while cross-linking consists of the immobilization of enzymes to each other into larger, macroscale

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aggregates. However, these immobilization strategies do not necessarily allow for the sophisticated spatial organization of multi-enzymes seen in cellular metabolic channeling, microcompartmentalization, etc.^{2,8,31–35} Therefore, a key challenge in achieving highly efficient artificial multi-enzymatic pathways is to develop new strategies to organize enzymes with high precision and predictability.

Deoxyribonucleic acid (DNA), the ‘blue-print’ of life, is one of the most important biopolymers as the primary carrier of genetic information in biological systems. In 1982, Nadrian Seeman proposed the idea of using this information carrying ability to construct immobile junctions using DNA with asymmetric sequences³⁶ through Watson-Crick base pairing³⁷ between complementary DNA strands, thus giving birth to the field of DNA nanotechnology. Over three decades after Seeman’s proposed idea, enormous amounts of different DNA-based nanostructures are emerging, ranging from bundles, nanotubes, 2D lattice arrays and 3D nanostructures.^{38–44} A breakthrough was made in 2006, when Paul Rothemund came up with the idea of folding long single-stranded M13mp18 genomic DNA (7249 bases) into arbitrary

2D shapes by a collection of short oligonucleotides, which are called ‘staple strands.’⁴⁵ These discrete structures with preprogrammed shapes and sizes are called ‘DNA origami.’ Within a few years of that innovation, the origami approach had been extended to create 3D nanostructures.^{46–52} Because of its predictability and programmability, DNA nanotechnology not only has been used to create outstanding, beautiful structures; these structures are also used extensively to organize other entities, such as noble metal nanoparticles, magnetic nanoparticles, quantum dots, carbon nanotubes, proteins, viral capsids and many other functional molecules.^{53–74} This fascinating bottom-up approach of DNA nanotechnology is especially useful due to the exceptionally high specificity and spatial addressability, making it a promising candidate to engineer numerous multienzyme cascade reaction.

In this review we shall discuss recent developments in organizing enzymatic pathways using DNA nanotechnology as well as the exciting challenges before us. In particular, we will cover techniques used to conjugate DNA to enzymes as well as discuss previous efforts in assembling multi-enzyme reaction pathways.



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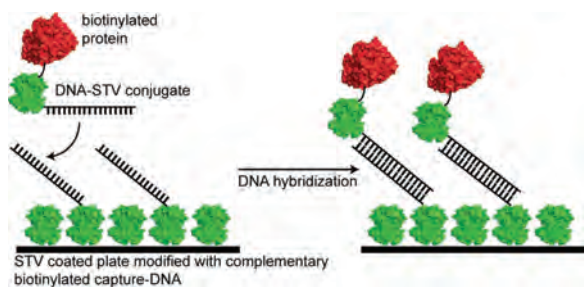
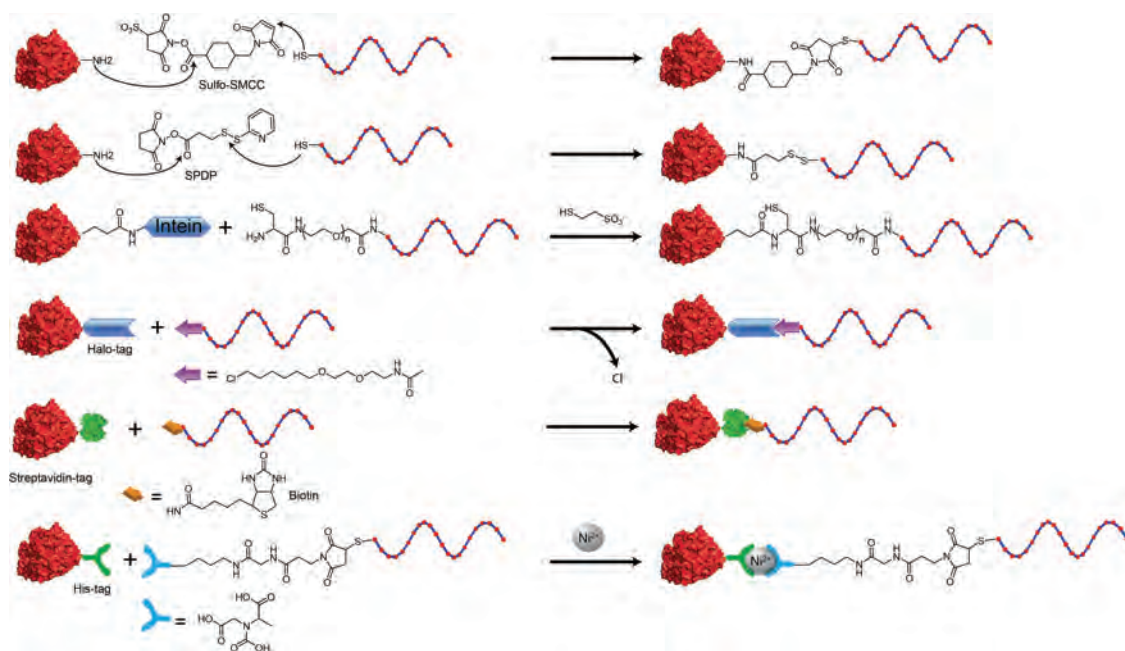


Figure 1. Schematic representation of DNA-directed immobilization of protein on a streptavidin (STV) coated microplate. Hybridization between oligonucleotides attached to the plate surface and those conjugated to streptavidin leads to target specific immobilization of the target protein (red) onto the surface.

2. DNA-ENZYME CONJUGATION METHODS

Development of DNA-protein coupling is one of the most crucial steps in assembling proteins on DNA nanostructures. The concept of DNA directed immobilization of enzymes was introduced almost twenty years ago by Niemeyer et al.^{75,76} Single stranded DNA and streptavidin were conjugated through covalent bonds using an amine-thiol bifunctional crosslinker. The DNA moiety of the hybrid macro-molecule provides a specific recognition capability to its complementary DNA, providing a means of using this complementary binding to achieve protein immobilization (Fig. 1). DNA: enzyme conjugation methods can be broadly separated into two categories—covalent and non-covalent attachment (Scheme 1).⁷⁷ For covalent conjugation, bifunctional cross-linkers containing thiol-reactive and amine-reactive

moieties are frequently used for DNA-protein conjugation due to the abundance of multiple of lysine and cysteine residues on the surface of proteins, as well as the ease of incorporating amine and thiol groups during commercial solid-phase DNA synthesis. Two very popular cross-linkers utilizing amine-thiol crosslinking are sulfo-SMCC (sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate)^{75,78} and SPDP (N-Succinimidyl 3-(2-pyridyldithio)-propionate).⁷⁹ However, the large number of available lysines and cysteines, which make this strategy attractive from an efficiency standpoint also severely limit the specificity of the approach. This presents a problem for applications in which site specific protein conjugation is required,⁸⁰ particularly those enzymes in which the lysine or cysteine residues are crucial for catalytic activity. Using genetic modification, site specific DNA-protein conjugates can be prepared using covalent and non-covalent interactions; for example, by mixing of biotinylated DNA with a protein genetically modified to contain a streptavidin domain, gives rise to non-covalent interaction.⁸⁰ Another example of a non-covalent site specific modification is the addition of reactive His-tag into proteins, which can interact with nitrilotriacetic acid (NTA) modified species.^{81,82} Thiol-modified DNA can react with maleimido-C3-NTA to create DNA molecules with specificity for His domain in the presence of nickel ions. While less frequently used, inteins,^{77,83} unnatural amino acids,⁸⁴ and HaloTags⁸⁵ provide even more options for site-specific covalent labeling of proteins with DNA (Scheme 1). More recent work has focused on finding site-specific modifications that can be accomplished without prior genetic modification of the



Scheme 1. Strategies for conjugating DNA to proteins utilizing both covalent (Sulfo-SMCC, SPDP, Intein-Tag, Halo-Tag) and non-covalent (Streptavidin-Biotin, His-tag) attachment methods.

protein. Gothelf et al. demonstrated a new technique for site-specific modifications, termed DNA-templated protein conjugation (DTPC), which utilizes non-covalent metal ion-coordination reactions to direct selective covalent bond formation in both genetically modified and wild-type proteins.⁸⁶ Francis et al. utilized a novel reagent, 2-pyridinecarboxaldehyde (2PCA), which can selectively modify the N-terminus of proteins without competition from lysine side chains.⁸⁷ This technique is particularly promising, as it could theoretically work for any protein with an accessible N-terminus.

3. ORGANIZATION OF ENZYMIC REACTION PATHWAYS USING DNA NANOTECHNOLOGY

One of the first examples of using DNA nanotechnology to engineer a multienzyme reaction pathway was the linear organization of nicotinamide adenine dinucleotide (NADH): Flavin mononucleotide (FMN) Oxidoreductase (NFOR) and Luciferase (Luc), which catalyzes two consecutive reaction steps, on double stranded DNA (Fig. 2(A)).⁸⁸ In this bi-enzymatic reaction cascade,

reduced FMN, produced by a reaction with NADH and catalyzed by NFOR, is used as a cofactor in the Luc-catalyzed oxidation of dodecanal. The spontaneous autooxidation of FMNH₂ is a competing side reaction that occurs if the product is not efficiently shuttled between the two enzymes. The DNA-directed organization was achieved by coupling biotinylated enzymes with DNA-streptavidin conjugates, which then hybridized to complementary DNA attached to the surface of a microplate. It was found that immobilizing the enzymes with direct proximity (Fig. 2(A), middle panel) enhanced the enzymatic activity almost 3-fold compared to the activity obtained from assembling the enzymes through random hybridization (Fig. 2(A), top panel). In 2009, Willner et al. reported the design of self-assembled DNA-strips attached with either two enzymes or a cofactor-enzyme pair.¹⁰ Two-hexagon and four-hexagon DNA-nanostructures, with the widths of ~13 nm and ~33 nm, respectively, were self-assembled and used to capture two enzymes, glucose oxidase (GOx) and horseradish peroxidase (HRP). In this bi-enzymatic pathway, GOx catalyzes the oxidation of glucose to gluconic acid, generating H₂O₂ as a byproduct. H₂O₂ can then be used as a substrate

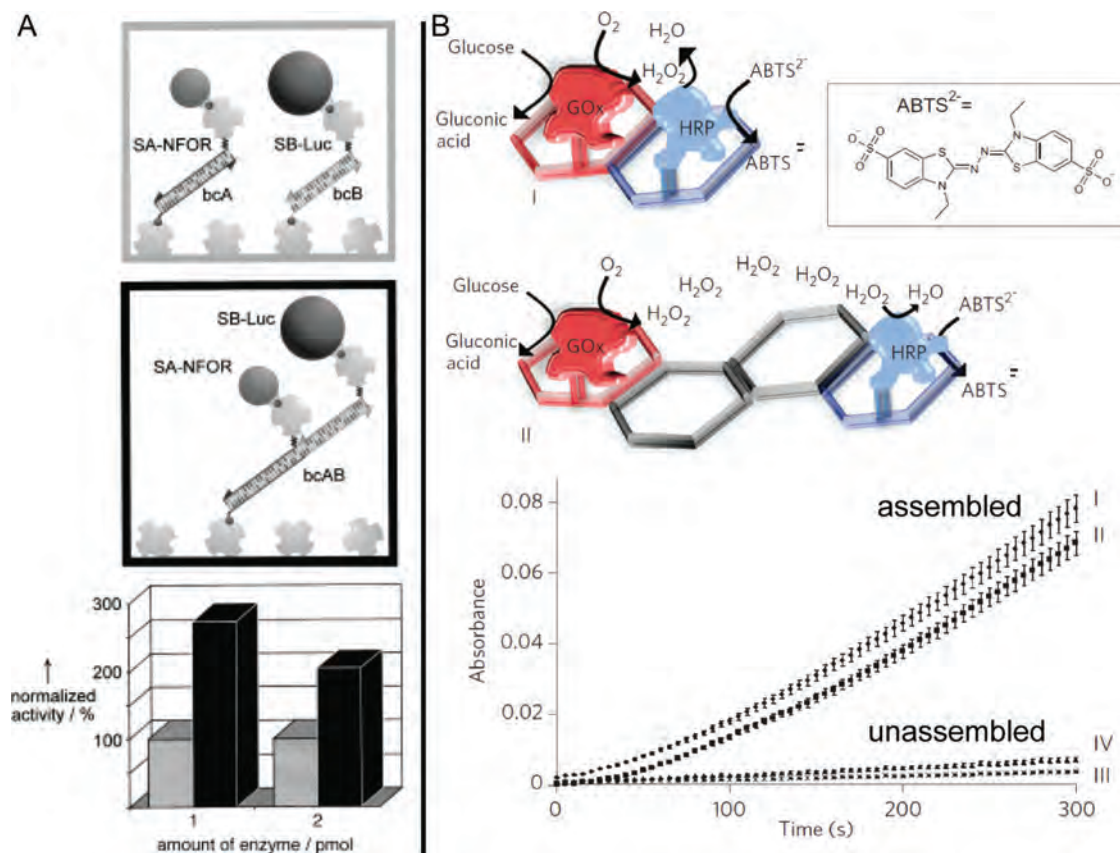


Figure 2. (A) Schematic representation of DNA-directed assembly of NFOR and Luc through random hybridization (top) and in direct proximity (middle), and the overall enzymatic activity at two different surface coverages (bottom) (Reproduced with permission from [88], C. M. Niemeyer, et al., *ChemBioChem* 3, 242 (2002). © 2002, Wiley). (B) Assembly of GOx and HRP enzymes on two dimensional DNA strips (top) and time-dependent absorbance changes showing the course of the reaction over time (bottom) (Reproduced with permission from [10], O. I. Willner, et al., *Nat. Nanotechnol.* 4, 249 (2009). © 2009, Nature Publishing Group).

for the HRP-catalyzed oxidation of 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic-acid] (ABTS^{2-}) to the colored product $\text{ABTS}^{\bullet-}$ (Fig. 2(B), top). Interestingly, in the absence of DNA scaffolds, the enzyme-cascade did not produce much $\text{ABTS}^{\bullet-}$ from ABTS^{2-} (Fig. 2(B), bottom), but immobilization of the enzymes on the DNA scaffolds led to a significant increase in the rate of the biocatalytic reaction. These results demonstrate that when enzymes involved in a cascade reaction are in close proximity, the local concentration of the intermediate, H_2O_2 , becomes very high, leading to a faster rate of the cascade reaction. In fact, the oxidation of ABTS^{2-} was ~ 1.2 -fold higher in the case of enzymes assembled on the two-hexagon structure compared to the four-hexagon structure, as the shorter distance between the enzymes limited diffusion of the H_2O_2 intermediate into the bulk solution and led to higher local concentrations. In a different approach, enzymatic activity was controlled by tuning the distances between two protein domains (Fig. 3(A)).⁸⁹ At first, the reductase domain (BMR) and the porphyrin domain (BMP) of cytochrome P450 BM3 were conjugated site-selectively to unique DNA oligonucleotides using the HaloTag approach. The DNA modified-BMR and -BMP were subsequently hybridized to a single stranded DNA template having complementary domains corresponding to each of the DNA strands attached to the proteins. The distance between the two domains was controlled by

hybridizing complementary oligonucleotides of different lengths to the loop present on the DNA template. The catalytic activity observed in the case of assembled structure was much higher compared to the unassembled domains. However, the enzymatic activity decreased upon increasing the distance between the domains.

A more detailed study into the distance dependence of the multienzyme cascade reactions was conducted by Yan et al. (Fig. 4).¹² The GOx and HRP enzyme pair used in previous works was coassembled on rectangular DNA origami tiles with four inter-enzyme distances: 10 nm, 20 nm, 45 nm, and 65 nm. Similar to the methods reported by Willner et al., the enzyme activities were measured in the presence of glucose and ABTS^{2-} by monitoring the absorbance change at 410 nm as $\text{ABTS}^{\bullet-}$ was produced. More than a 15-fold increase in the cascade activity was observed for the enzyme assembly with 10 nm inter-enzyme distance compared to the activity of unbound enzymes (Fig. 4(A)). A drastic decrease in the enzyme activity was observed for the sample with 20 nm inter-enzyme distance and then there was a gradual decrease in the activity with further increase in inter-enzyme distances. The exceptionally high activity observed at the 10 nm inter-enzyme distance was attributed to the “dimensionally restricted diffusion along protein surfaces.” To prove this hypothesis, the authors constructed a protein bridge between GOx and HRP to

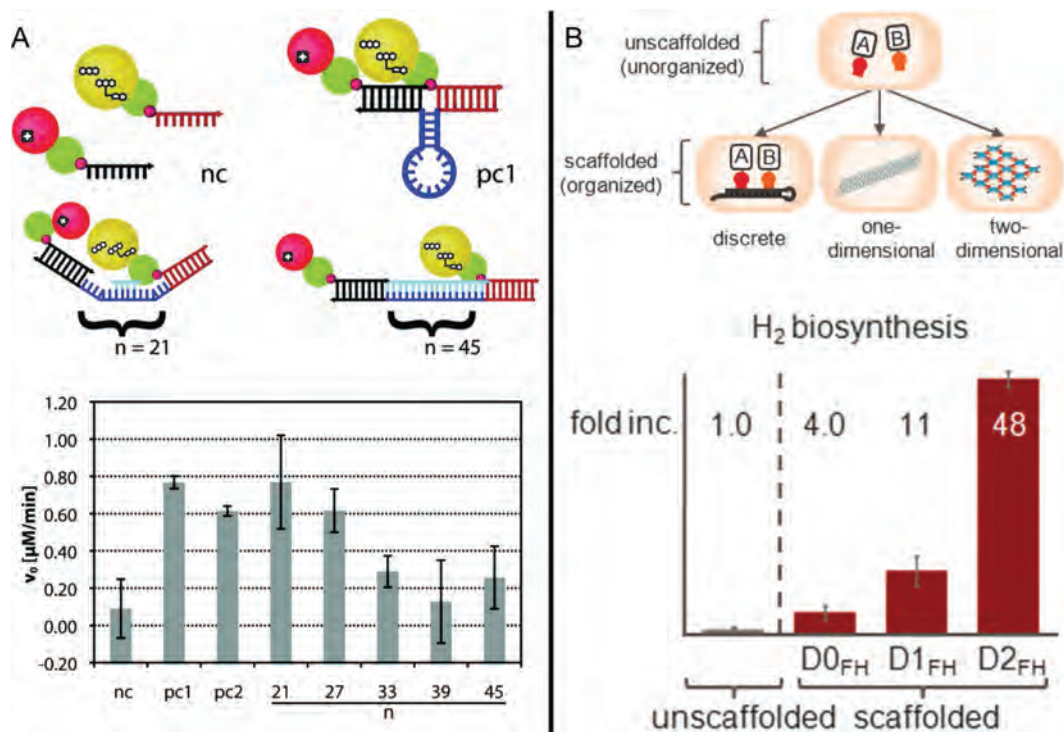


Figure 3. (A) Hybrid DNA-Cytochrome P450 BM3 devices with variable distances between the reductase domain BMR and the porphyrin domain BMP (Reproduced with permission from [89], M. Erkelenz, et al., *J. Am. Chem. Soc.* 133, 16111 (2011). © 2011, American Chemical Society). (B) Hydrogen biosynthesis by assembling hydrogenase and ferredoxin *in vivo* using discrete, 1D, and 2D RNA nanostructures (Reproduced with permission from [11], C. J. Delebecque, et al., *Science* 333, 470 (2011). © 2011, AAAS).

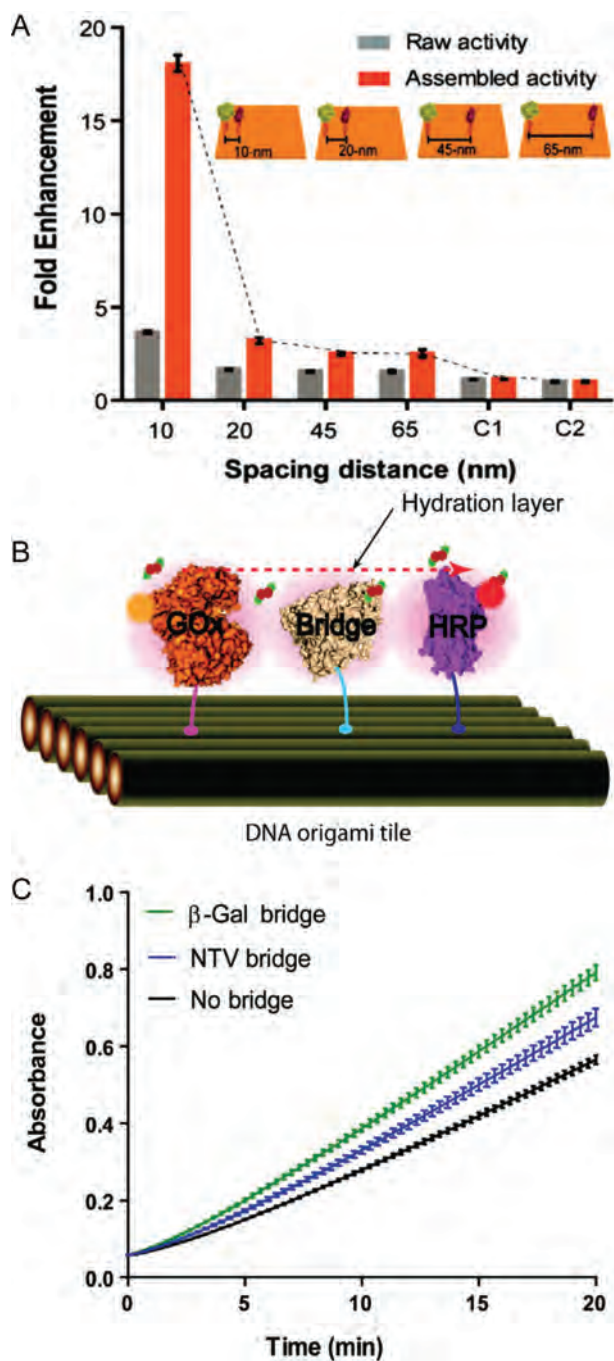


Figure 4. Distance dependence study of inter-enzyme cascade reaction with a bridging protein enabling surface-limited H_2O_2 diffusion between enzymes (Reproduced with permission from [17], J. Fu, et al., *Acc. Chem. Res.* 45, 1215 (2012). © 2012, American Chemical Society and from [12], J. Fu, et al., *J. Am. Chem. Soc.* 134, 5516 (2012). © 2012, American Chemical Society).

connect the hydration shells of the enzymes, while placing them 30 nm apart on the DNA origami tile (Fig. 4(B)). A $\sim 42\%$ enhancement in raw activity was observed with the assembled GOx/HRP pairs having streptavidin-conjugated β -galactosidase (β -Gal) as a noncatalytic bridging protein, compared to the activity observed for assemblies

without a bridge (Fig. 4(C)). Furthermore, only a $\sim 20\%$ enhancement in the raw activity was observed for assembled GOx/HRP pairs bridged by neutravidin (NTV), which has much smaller diameter (~ 6 nm) compared to that of β -Gal (~ 16 nm), signifying that a larger bridging protein filled the space between GOx and HRP more and improved the overlap of the hydration shells of the enzymes. Theoretical work on the benefits of “metabolic channeling” through the use of restricted diffusion in multi-enzyme systems support these results, while providing further insight into the mechanisms and limitations of these concepts.^{90,91}

While a considerable amount of effort has been spent studying how enzyme-scaffold interactions affect the efficiency of the catalytic system, very little work has been done studying how substrate-scaffold interactions alter the kinetics of the enzymes. Wheeldon et al. used a system consisting of 1, 2, or 3 HRP’s bound to a triangular DNA scaffold, and measured the kinetics of the catalysis using different substrates.⁹² The authors demonstrated that DNA-scaffolded enzymes mimic the Sabatier principle; substrates with very low or very high affinity to the scaffold showed only minor improvement in enzyme kinetics (quantified with Michaelis-Menten constant and turnover number), while substrates in the “sweet spot” demonstrated faster kinetics. The authors contribute this to the fact that substrates with low affinity to DNA will not enhance local concentrations near the enzymes, while substrates with a very high affinity will bind strongly to the DNA and be unavailable for catalysis. This work highlights the importance of taking into account substrate/DNA interactions when designing and analyzing multi-enzyme systems built using DNA scaffolds.

The studies discussed to this point have investigated the dependence of enzyme activity on interenzyme distance in a static manner. But cellular pathways often show switchable on-off activities, which are critical to many metabolic functions.^{93–95} In work by Yan et al., a dynamic DNA tweezer nanostructure was developed to regulate enzyme activity.⁹⁶ The DNA tweezer actuates the enzyme function by switching between open and closed states (Fig. 5(A)). The enzyme and the cofactor were conjugated to the two arms of the tweezer, which was opened by hybridizing the regulatory loop with a complementary DNA strand. The enzyme activity was detected by observing the fluorescence of resorufin, which was produced by the reduction of resazurin in the presence of NADH. Figure 5(B) shows the efficient regulatory cycling of the enzyme/cofactor pair assembled on DNA tweezers. A 5-fold enhancement in the catalytic activity in closed state compared to the open state is consistent even after four cycles, demonstrating the reliability of such artificially engineered enzyme actuators. In another report, Yan et al. successfully demonstrated an artificially engineered swinging arm’s applicability toward a multi-enzyme catalytic process.¹⁴ Such swinging arms or covalently attached prosthetic groups are

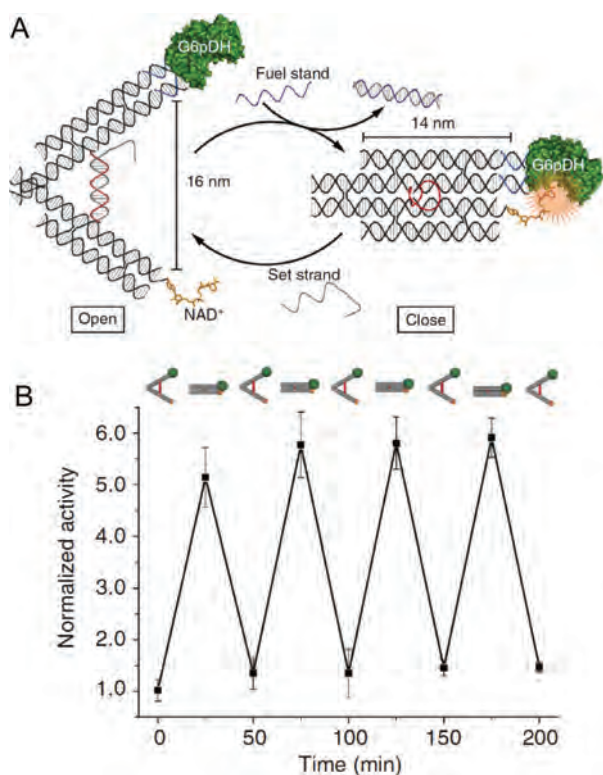


Figure 5. Schematic of G6pDH/NAD⁺-assembled DNA tweezers (A) with enzyme activity assay (B) (Reproduced with permission from [96], M. Liu, et al., *Nat. Commun.* 4, 2127 (2013). © 2013, Nature Publishing Group).

essential to multitude of multienzyme systems.⁷ Mimicking of this process was achieved by using DNA nanotechnology, in which a DNA double-crossover tile was utilized to assemble enzymes glucose-6-phosphate dehydrogenase (G6pDH) and malic dehydrogenase (MDH). By utilizing a single stranded DNA conjugated to NAD⁺ as a swinging arm placed equidistance between the two enzymes, the activity observed was ~90-fold higher than that obtained from the DNA-enzyme complex with free NAD⁺ in solution. By increasing the ratio of MDH and the NAD⁺-modified swinging arm to G6pDH up to 4:1, a ~280-fold enhancement in activity was observed. One of the interesting characteristics of this multienzyme cascade with a swinging arm is the specificity of the swinging arm toward an enzyme assembled in a close proximity compared to a freely diffusing enzyme. This hypothesis was investigated by adding another enzyme, lactate dehydrogenase (LDH) that competes with MDH for NADH, into the system (Fig. 6(A)). It was found that the MDH activity in the G6pDH-NAD⁺ assembly with freely diffusing LDH and MDH was very low as there was a strong competition between the two enzymes for NADH. However, the activity increases gradually with the increase in the percentage of the G6pDH-NAD⁺-MDH assembly in the solution; MDH can now access the NADH more easily owing to its proximity toward the swinging arm (Fig. 6(B)).

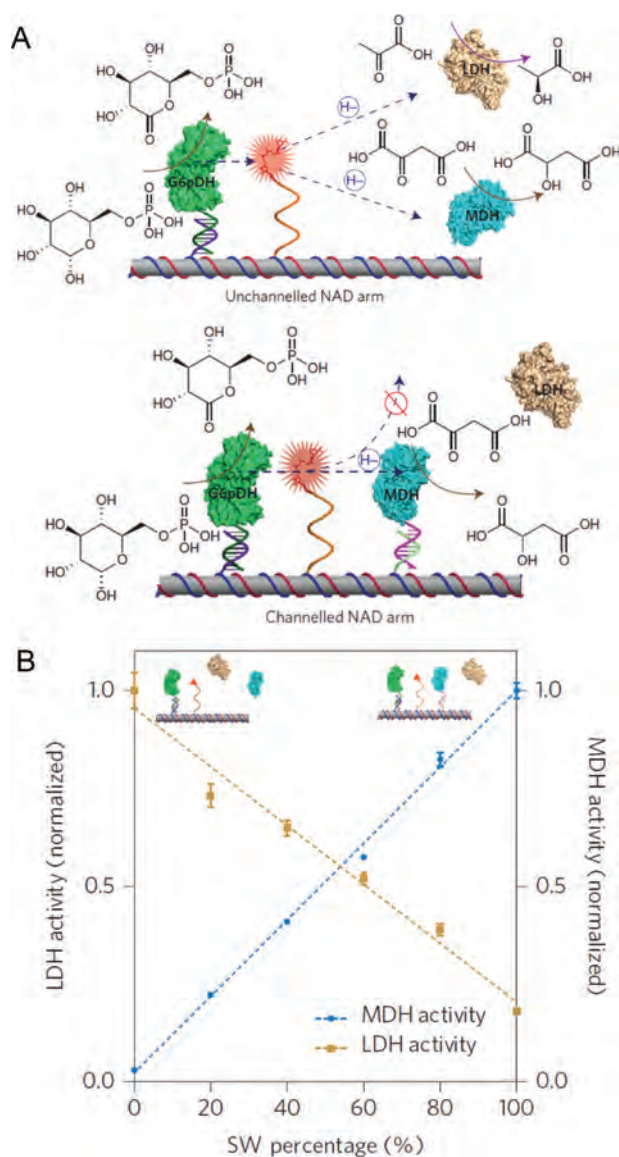


Figure 6. (A) Schematic of swinging arm assembly showing specificity toward assembled MDH in the presence of a competing LDH. (B) The normalized activity assay of MDH and LDH with the change in the percentage of G6pDH-NAD⁺-MDH assembly (Reproduced with permission from [14], J. Fu, et al., *Nat. Nanotechnol.* 9, 531 (2014). © 2014, Nature Publishing Group).

One of the first demonstrations of engineering a multienzyme reaction *in vivo* was reported by Silver et al.¹¹ Synthetic RNA molecules assembled into either discrete pairs, 1D, or 2D scaffolds were used to control the spatial organization of [FeFe]-hydrogenase and ferredoxin (Fig. 3(B), top), an enzyme pair which together catalyze the reduction of protons to hydrogen.^{97,98} Compared with unscaffolded enzymes, the discrete, 1D, and 2D RNA assemblies resulted in ~4-, ~11-, and ~48-fold increase in hydrogen biosynthesis respectively within a bacterial system (Fig. 3(B), bottom). These remarkable results have opened up a new direction of *in vivo* metabolic engineering

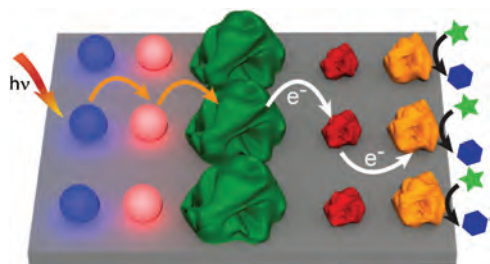


Figure 7. Schematic representation of artificial photosynthetic-multienzymatic nanoreactor. Using DNA nanotechnology, photon acceptors (blue and pink) can be patterned so that efficient energy transfer can occur, leading to a chemical reaction in the photosynthetic core (green). Induced proximity of downstream enzymes (red and yellow) allows for directed movement of the generated electrons and efficient catalysis of substrate (green star) to final product (blue hexagon).

using nucleic acid-based nanoreactors. The Silver group has further expanded on this work, using 1D and 2D RNA nanostructures assembled inside *E. Coli* to direct the organization of three different enzymatic pathways.⁹⁹ At first, a bi-enzyme system consisting of the acyl–acyl carrier protein reductase (AAR)/aldehyde deformylating oxygenase (ADO) enzyme pair was used to catalyze the formation of alkanes from fatty acyl–acyl carrier protein precursors. Enhanced production (~80%) of the alkane was discovered using 1D and 2D RNA scaffolds over co-expression without scaffolding. The production could be further modulated by controlling geometry of the scaffolding via different RNA tile designs, leading to enhancements from 40%–140%. The paper also demonstrated the successful production of three enzyme and four enzyme systems utilizing the same techniques. Another work by Lu et al. engineered various bi- and tri-enzymatic systems that could be used in an *in vivo* murine model.¹⁰⁰ Enzymes were assembled using DNA oligonucleotides conjugated to specific inhibitors of the enzymes and then assembled into duplexes or 4-way DNA junctions. Following co-incubation of the DNA scaffold and enzymes, the nanoconjugates were encapsulated inside polymer shells and then the inhibitor DNA-scaffold removed. While several enzymatic cascades were investigated, the most promising involved the use of alcohol oxidase and catalase; this system demonstrated improved alcohol metabolism in a live mouse model. While not utilizing the complex DNA structures seen in other works, these results point to a promising future in clinically relevant applications for scaffolded-enzyme systems.

4. CONCLUSION AND PROSPECTS

The various enzyme assembly systems discussed throughout this review share a common phenomenon that is frequently observed in natural systems—the assembled enzymes show much higher activity than free enzymes due to the restricted diffusion and improved channeling of

substrates. As DNA nanotechnology advances, it will be possible to create artificial bioreactors mimicking nature that were previously unattainable. The ability to engineer multi-enzyme assemblies with a high degree of spatial control would have vast array of applications ranging from biosynthesis to therapeutics to sensors and many more. At the same time, these systems will enable us to study the effect of many parameters, such as distance and orientation, on the multi-enzyme cascade process. Furthermore, with the advent of DNA-based artificial photonic devices and engineered enzyme assemblies, we can envision a day in which the combination of the two technology will be helpful to create devices for sustainable and renewable energy as well as light driven biocatalysis (Fig. 7). The applicability of these enzyme systems will hopefully drive scientists throughout the world to improve on their performance and continue to explore more elaborate and complex designs.

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