Cascaded Enzyme Reactions over a Three-Dimensional, Wireframe DNA Origami Scaffold

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ABSTRACT: DNA nanotechnology has increasingly been used as a platform to scaffold enzymes based on its unmatched ability to structure enzymes in a desired format. The capability to organize enzymes has taken many forms from more traditional 2D pairings on individual scaffolds to recent works introducing enzyme organizations in 3D lattices. As the ability to define nanoscale structure has grown, it is critical to fully deconstruct the impact of enzyme organization at the single-scaffold level. Here, we present an open, three-dimensional (3D) DNA wireframe octahedron which is used to create a library of spatially arranged organizations of glucose oxidase and horseradish peroxidase. We explore the contribution of enzyme spacing, arrangement, and location on the 3D scaffold to cascade activity. The experiments provide insight into enzyme scaffold design, including the insignificance of scaffold sequence makeup on activity, an increase in activity at small enzyme spacings of <10 nm, and activity changes that arise from discontinuities in scaffold architecture. Most notably, the experiments allow us to determine that enzyme colocalization itself on the DNA scaffold dominates over any specific enzyme arrangement.

KEYWORDS: DNA nanotechnology, enzymatic cascades, self-assembly, biomolecular scaffolds, DNA origami
biochemical reactions based on peroxidase activity and a H$_2$O$_2$ intermediate.

The majority of DNA-enzyme scaffold work has utilized 1D or 2D scaffold designs,$^{12,15,27,28,30}$ with many finding that smaller interenzyme spacing leads to higher activity. However, to reveal the mechanisms involved and for enabling future designs, it is highly important to explore the behavior of enzymatic cascades on a 3D scaffold, as both substrate channeling and solution diffusion can take place. From the design perspective, a 3D scaffold enables a greater organizational space and balancing of interenzyme substrate exchange through solution diffusion and potential channeling induced by the scaffold. Moreover, a 3D architecture allows exploration of enzyme placement in relation to each other beyond the planar manner found on 2D surfaces. The significance of enzyme-DNA origami binding location on enzyme activity was recently demonstrated, revealing that local environment can be impactful.$^{26}$ Though recently semi-enclosed structures$^{37,39}$ have been used in enzymatic catalysis and topological structures$^{40,41}$ in enzyme pairings for electrochemical detection, systematic study is required to reveal emergent properties arising from organizing enzymatic cascades on 3D scaffolds. The importance of reaction analysis over 3D structure becomes even more critical in mesoscale assemblies of interlinked DNA structures. For example, 1D and 2D mesoscale assemblies have been explored with DNA-functionalized enzymes,$^{15,30}$ and an ability to form 3D organizations of enzymes was shown, both in lattices of DNA frames$^{42}$ and in inorganic scaffolds such as metal–organic frameworks.$^{43}$ Recent developments in dynamic nanoassembly also highlight the need to understand the impact of local environment and organization on enzyme activity.$^{36,44,45}$

A 3D wireframe DNA origami geometry enables a versatile addressable space that allows exploration of how a two-enzyme cascade is dependent on the enzyme arrangement, both in relation to each other and to scaffold structure, including spacings and binding locations. In this study, we explore these factors and their relative importance by using a library of DNA scaffolds based on a 3D DNA frame topology that allows for (1) the ability to vary enzyme binding locations over different wireframe edges and at different relative positions to each other, (2) a large testable edge length, enabling spacing tests outside the size regime of the enzymes (>5 nm), and (3) a discontinuous scaffold structure in order to study its effects on substrate channeling. We utilize general and widely used enzyme functionalization and attachment methods$^{11,14,26,28,30}$ to explore these effects by varying enzyme binding locations on a 3D scaffold while allowing for all possible orientations of enzyme relative to the attached strands. This approach provides a systematic study over averaged enzyme rotation conformations for well-defined positions on 3D scaffolds. We investigated and compared reactions for 42 spatial arrangements of GOx and HRP cascades, as discussed below.

**RESULTS AND DISCUSSION**

Scaffold Construct

The selected 3D wireframe topology, a DNA octahedron shown in Figure 1A, is formed through DNA origami assembly (detailed in Methods) with a design linking 12 high density...
DNA bundles, or struts, each acting as an edge of the octahedron. Each strut consists of six double-stranded DNA helices, and thus staple strands forming the struts (listed in Table S1) provide addressability lengthwise as well as circumferentially around each strut. Throughout this work, we maintain a consistent naming scheme through assigned vertex letters and struts defined by the vertices they connect; that is, strut AB runs between vertices A and B, as shown in Figure 1A, which provides a “map” for the library of all formed cascades. Vertices A–D cover the planar x−y symmetry of the structure with vertices E and F providing the z-directional vertices. These designations are “absolute coordinate” designations in that they correspond to specific vertices in the origami design, the layout of which is shown in Figure S12. This design allows us to prescribe enzyme binding site locations, offering access to external and internal “spaces” of the origami frame in which to localize enzymes, a testable edge length of ∼30 nm, discontinuities in the scaffolding DNA bundle structure due to vertices, and varied binding layouts and relative enzyme positions on a fixed scaffold topology.

Using this same wireframe, we created libraries of origami with differing binding site combinations for glucose oxidase and horseradish peroxidase placement (Figure 1B). This approach allows us to systematically investigate the influences of enzyme arrangement on coupled enzymatic function for scaffold-attached GOx and HRP. A cascaded enzymatic reaction of GOx and HRP, as seen in Figure 1A, produces a measurable fluorescent product Resoru in from a glucose primary substrate. In this work, we have investigated 42 combinations of enzyme arrangements, as specifically discussed below. Functionalization of the enzymes with single-stranded DNA (ssDNA) allows them to hybridize to complementary ssDNA on each origami design. Functionalization was undertaken by first mixing enzymes with sulfo-EMCS, which binds to primary amines, and then mixing with thiolated ssDNA.1,16,20,28,30 This attachment chemistry is site nonspecific for the enzyme, thus in the ensemble of formed structures we explore a behavior averaged over enzyme orientation relative to the DNA linking it to origami. This design both allows us to directly compare enzyme arrangements on a 3D scaffold without intervening effects of fixed rotational orientation and compare our results to previous studies for 1D and 2D scaffolds with similar methods of enzyme functionalization. Further details of enzyme modification are provided in Methods and in the SI, where DNA functionalization of the enzyme causes a reduction in glucose oxidase and horseradish peroxidase activity of 10% and 40%, respectively (Figure S1). Since all studies throughout this work utilize DNA-functionalized enzymes, the results are direct comparisons of the same enzyme sets regardless of the effect of DNA-functionalization.

Figure 1 demonstrates the prescribed attachment of enzymes onto the origami structure and their binding using sequence-prescribed DNA hybridization. We introduce the naming scheme that details binding location on the origami topology based on vertex and strut identification. Methodology for TEM imaging and visualization of GOx binding to origami in Figure 1A is was conducted according to previous literature.22 Both the DNA-enzyme conjugation chemistry and conjugate hybridization to the scaffold use techniques that have been

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extensively utilized and documented in the literature, capable of achieving high scaffold binding efficiencies up to 80–90%.11,18,26,28,30 TEM imaging in Figure 1 and Figure S3 demonstrates that enzyme incubation did not have a visible structural effect on the origami, and glucose oxidase can be visualized in Figure 1A, ii. However, a quantitative assessment of binding efficiency using imaging methods (APM and electron microscopy) is problematic for a 3D wireframe origami. To quantify enzyme-origami binding, we used fluorescence methods using a fluorophore-quencher pairing. In our system, the DNA-functionalized enzymes possess a quencher, and the ssDNA origami binding site is labeled with a fluorophore. Binding efficiency was measured by comparing the enzyme attachment conditions in this study to saturated binding sites. Two different origami were studied using this approach (Figure S2) to evaluate binding to sites on a strut near a vertex and far from it. Glucose oxidase showed a binding efficiency of approximately 70–74%, while horseradish peroxidase exhibited a higher binding efficiency of approximately 90–94%.

**ENZYME COLocalIZATION ON A WIREFRAME SCAFFOLD INCREASES REACTION RATE**

The first experimental system examined whether arbitrarily prescribed enzyme colocalization on an octahedron origami affects the rate of product synthesis as compared to delocalized enzyme control systems, shown in Figure 2A. We functionalyzed the octahedra with two binding sites, each specific to either the glucose oxidase or horseradish peroxidase (sequence design listed in Table S2). The horseradish peroxidase is located at vertex E (in the absolute coordinate system shown in Figure 1A), oriented toward the external space with glucose oxidase oriented toward the internal space on Strut AD, parallel to the ABCD plane. Experimental controls are used to compare the reaction rates of this scaffold-colocalized scheme, denoted as reaction i (Figure 2A, i), to systems that physically decouple the binding locations of the two enzymes. The first control consists of the two enzymes, bound to their respective binding sites on the octahedra, but on two separate frames rather than colocalized on the same origami (Figure 2B, ii). As this control requires a total origami concentration at double the concentration of each of the enzymes individually, a nonbinding origami is added to the colocalized enzyme reaction i in order to maintain the same origami concentration. A second control (Figure 2B, iii) consists of the same enzyme set placed in solution with an equivalent amount of nonbinding origami. In order to present the most accurate comparison, we have maintained an equivalent concentration of origami and enzyme in each reaction.

Two effects of colocalization can be clearly noted from the product concentration kinetic plot in Figure 2B. First, the activity of the colocalized setup, system i, is approximately 2-fold greater than that of control system iii and 50% greater than setup ii. The time needed to reach this maximum reaction rate is shorter than both controls. In order to remove the possibility that the higher colocalized activity is dependent on the specific reporting reaction (Amplex Red → Resorufin catalyzed by HRP), we used a different reporting molecule to track activity. Figure 2B, inset shows systems i and ii using a different reporting reaction, ABTS → ABTS’+, tracked through absorbance measurements at 420 nm. The lack of initial readings is due to the reduced sensitivity of this assay, but it provides a measure of the maximum reaction rate once a measurable threshold is reached. The ABTS reaction shows an even greater difference between reactions i and ii, where the colocalized enzyme sample yields nearly double the rate of delocalized, scaffold-bound enzymes. In plotting turnover rate versus glucose concentrations in Figure 2C, we can pull out apparent Michaelis–Menten parameters, where the difference in $k_{cat}$ values (66/s vs 44/s) are clearly seen between colocalized or separately bound systems, but a more minor difference is observed in $K_m$ values (7.2 mM vs 9 mM). In a one enzyme setup, this would provide evidence that the rate of catalysis is influenced by the colocalization as opposed to binding kinetics, but this explanation is limited by the application of Michaelis–Menten kinetics to a two-enzyme cascade.

Control reactions ii and iii are designed to elucidate the specific influence of enzyme attachment to scaffold in order to separate this contribution from colocalization effects. In deciphering where the difference between these setups may come from, it is also informative to test the enzymes separately to gain a fuller sense of how origami attachment affects the individual half reactions. Thus, two setups were run for each enzyme, whereby GOx and HRP were incubated in their own reaction volume with either free origami in solution or with origami containing enzyme binding sites. For the glucose oxidase half-reaction, a 100:1 excess of free HRP (unfunctionalized enzyme) was added to the solution to ensure that GOx was the rate-limiting reaction. An increase in activity is measured for glucose oxidase attachment while a smaller increase is noted for horseradish peroxidase, as shown in Figure S8. The analysis presents HRP as the enzyme possessing a lower turnover rate than GOx by nearly 10x, which is in general agreement with previously published work.15,27,28 Thus, HRP acts as the rate-limiting enzyme and an overall reaction rate would be most affected by changes in this enzyme’s activity.

The increase in activity for both enzymes upon scaffold binding can explain elevated activity seen in reaction ii versus reaction iii in Figure 2. This result has been demonstrated by other works also showing higher enzymatic activity on DNA scaffolds,16,17,26,27 potentially attributed to locally lower pH near the scaffold18,26 or substrate/intermediate quarantining in a scaffold hydration layer acting as a “virtual compartment”.5,15–17,27,28 Our own work has shown such an effect for glucose oxidase,26 yet this still does not sufficiently explain colocalization results themselves. The apparent $k_{cat}$ of the coupled reaction, as compared to the rate limiting horseradish peroxidase alone (Figure S8) in the same scaffold placement, is more than double the rate (66/s vs 26.5/s, respectively). Similar colocalization effects have been seen in enzyme arrangements of glucose oxidase, horseradish peroxidase, catalase, and β-galactosidase, among others, using linear scaffolds, DNA sheets, and enclosed cubes.12,14,28,30,46 However, more complex structural and geometric considerations have not been heavily considered over existing 2D DNA scaffolds, where DNA density is consistent over the structure and enzyme attachment points are located on the same region of scaffold. In studying these considerations, we are not making an assertion about absolute enzyme activity in any bound or unbound format but rather across enzymes bound to a given structure in a library of arrangements, as shown in Figure 1B and discussed below.
Relative Enzyme Placement Is Not a Significant Contributor to Enzymatic Activity Increase

This colocalized layout leads to a higher reaction rate, yet it is just one enzyme arrangement possible on the 3D octahedron wireframe. We further probed effects of relative enzyme placement and absolute binding position at a given spacing, enabled by our rigid but “open” 3D scaffold. Whereas enzyme orientation is often discussed in the context of rotation around the center of the enzyme itself, here we consider enzyme arrangements as pertaining to the relative positions of the enzyme centers with respect to each other while averaging over all orientations. We aimed to keep the distance between enzyme binding locations approximately the same, in the range of the octahedron strut length (≈30 nm), and beyond the hydration layers associated with each of the enzymes.12,28,47 Thus, results should shed light on reaction behavior outside of...
spacings dominated by direct proximity phenomena. Establishing an arrangement library thus helps to determine if elevated activity arises from specific interactions or channeling mediated by the enzyme arrangement itself without contributory scaffold influences.

In order to test the effect of 3D enzyme arrangements on reaction rate, a library of binding-strand layouts over 12 combinations of enzyme arrangements was assembled, as presented in Figure 3A,B (full sequence descriptions are listed in Table S3). This library covers various cascade architectures for the DNA octahedron topology, namely relating to combinations of external–internal (Group EI) and internal–internal (Group II) enzyme arrangements where 3D layouts may affect the reaction. We note that changes in layout can influence the interenzyme distances, but given the geometry of struts and distances involved, this contribution was neglected in our design considerations. The octahedron regions of Vertices A and E are utilized with external and internal binding positions used at or near Vertex E, and three different struts extruding from the interior region of Vertex A to control relative placement of the enzyme at this location, yielding distances between binding locations of approximately 25–30 nm. Variations of these classes using reversed enzyme locations by switching GOx and HRP (Groups EIR and IIR) are also included to address any possibility that local DNA sequence and structure induce further local effects over the origami structure or on the enzymes themselves.

Figure 3C presents reaction rates for each of the enzyme arrangements presented in Figure 3B with results presented in the linear range of the reaction. The plot shows that product synthesis falls within a reaction rate range of 0.091 to 0.102 μM/s of Resorufin over the library setups. This result demonstrates that colocalized activity over this library of enzyme arrangements does not yield significant differences in reaction kinetics. Such a conclusion is supported by previous modeling of 2D-scaffolded enzymes, where the radial distribution function around enzymes is radially symmetric and thus should not express orientation-specific behavior.48

Significantly, in comparing Groups EI and II to their respective reversed enzyme counterparts in Groups EIR and IIR, we can also conclude from the plot that the specific scaffold sequence makeup at a given enzyme attachment point did not play a role in overall activity. Lastly, the reaction rate of this library presents double the reaction rate of the colocalized setup Figure 2 (reaction i), which is expected given the doubling of enzyme concentration in this experimental setup. A higher

Figure 4. Spacing enzymes across a three-dimensional frame scaffold. A library of scaffold-enzyme binding layouts is created to explore reaction differences between enzymes bound on the same octahedron strut or adjacent struts over a range of enzyme spacing with similar binding orientations on the struts. (A) Schematic of the struts used in this experimental setup with a fixed vertex binding location for one enzyme at Vertex A of Strut AD. Placement of the binding strand at each binding location, with the exception of enzyme binding at the vertices (which applies to fixed binding site and the 30 nm spacing location), is designed to maintain consistency in enzyme binding orientation to the bound strut across different spacings. (B) The range of enzyme spacings tested. (C) Fluorescent product synthesis after a 10 min reaction based on the nature of strut binding and enzyme spacing upon addition of 150 mM glucose to a DNA origami/GOx/HRP concentration of 0.2 nM and Amplex Red concentration of 200 μM. A 600 s reaction time was selected to ensure reactions were in the linear phase, with full plots shown in Figure S11. All fluorescence results consist of the average and standard deviation from eight measurements at each spacing, where four measurements are replicates with glucose oxidase at Vertex A of Strut AD, and the other four are replicates with the enzyme positions switched. The full structure library is presented in Table S5 with results between these two subsets of switched enzyme binding shown in Figure S10.
enzyme-origami concentration was used across larger library setups to ensure that experimental variability arising from very low enzyme concentration would be minimized across samples and plate wells.

**Spacing Effects Arise from Both Proximity and Scaffold Design**

We varied enzyme spacing over the 3D wireframe scaffolds while controlling for relative enzyme placement to create a spacing library, as shown in Figure 4. However, as opposed to 2D sheets,12,15,27,28 or tubes,39 our scaffold naturally possesses structural discontinuities at each of the six vertices where densely linked six-duplex DNA struts are linked together through four single-stranded bases, which provide a loose arrangement of DNA at each vertex. This may significantly affect a substrate channeling mechanism due to reduced charge density and structural discontinuity. Thus, we take into consideration the scaffold structure between the two spaced enzymes when building our library, invoking a naming scheme of “same-strut” and “different-strut” to differentiate between the locations of the ssDNA overhangs that act as enzyme binding linkages. This differentiation has been introduced based on conclusions from modeling studies, which have suggested that the polyamionic nature of the DNA can create a boundary layer that presents a locally lower pH due to the tendency for the high-density anions in DNA origami to attract counterbalancing H+17,18. While capable of affecting enzyme activity itself,26 it is also capable of enhancing the local concentration of intermediates,28,29 and such a boundary could be disrupted by structural discontinuities. It should be noted that all enzyme arrangements explored in Figure 2 and Figure 3 correspond to different-strut arrangements.

We employed a systematic study to determine how these effects manifested on our open, 3D wireframe scaffold, composed of loosely connected, stiff individual DNA struts. In this manner, we can view each of the struts as nearly separate origami, loosely connected to neighboring origami. Thus, if substrate channeling is present and playing a role in activity, we may be able to determine whether transport acts differently over a single, continuous structure versus a discontinuous, complex structure where breaks in features at the vertices may interfere with channeling. The experimental approach for the spacing library is summarized in Figure 4A,B with full sequence descriptions listed in Tables S4 and S5. We fixed an enzyme binding location at the terminus of a selected strut (Strut AD, Vertex A) and then modulated the distance of the second enzyme’s binding location along the length of either the same strut or a different, neighboring strut. By fixing the location of one enzyme at the vertex of the octahedron frame, we aimed to provide the same local environment for this vertex-bound enzyme in order to minimize local environment contribution and specifically analyze the pairing of the enzyme linkages in the same-strut and different-strut arrangements. The local environment contribution is further removed by switching the positions of the enzymes, so that every spacing is analyzed both with GOx and HRP at the vertex location. Importantly, because enzyme placement on the 3D scaffold can be altered even at the same spacing, we selected binding locations to minimize binding orientation differences between locations. Figure S13 shows the locations of enzyme binding on the same-strut and different-strut systems, where binding direction is predominately selected to face a 180° region on struts AD and AE toward the exterior of the structure.

In order to determine that the geometric nature and rigidity of the scaffold led to actual nanoscale spacing values that corresponded to our designs, we utilized a Förster Resonance Energy Transfer (FRET) method to determine relative spacing between the 2.5, 5, and 10 nm (the upper limit for FRET) of nominal designed interenzyme separations on the associated octahedra. Cy3 and Cy5 fluorophore-labeled ssDNA were used to bind the positions, corresponding to the same DNA sequences as those used to functionalize the GOx and HRP. The results, shown in Figure S9, demonstrate that the measured FRET trend and values (5.75 and 8.65 nm, using the 2.5 nm sample as a spacing standard, vs 5 and 10 nm for designed samples respectively) are in good agreement with the designed spacings (see Supporting Information for details). In interpreting these results, we can also gather that despite the flexible nature of the enzyme-DNA linkage, the binding distances are generally held.

As seen in Figure 4C, both same-strut reactions and different-strut reactions show a moderate increase in activity at approximately 5 nm. Activity decreases at distances smaller than 5 nm, most likely due to steric hindrance imposed by the neighboring enzyme, an effect seen in previous literature.28 While overall behavior between the two setups is similar, differences at the larger (>15 nm) interenzyme spacings are observed as the same-strut reactions retain a higher activity while different-strut reactions trend lower. As mentioned earlier, these plots account for any potential differences in local DNA environments on enzyme activity, where each plotted point on Figure 4C is an average of two scaffold results with the enzyme identity at each binding location switched. Figure S10 breaks down these results further, specifically looking at the measurement subsets with either glucose oxidase or horseradish peroxidase at Vertex A (the full structure library is presented in Table S5). Local sequence differences at each location do not significantly influence cascaded activity, matching this conclusion from the enzyme arrangement study presented in Figure 3.

These overall results may arise due to two different effects seen within DNA-scaffolded systems, where evidence for substrate channeling has been observed both between closely spaced enzymes12,28,47 and in instances where scaffold contributes to increased coupled activity.5,15,16,27,31 In comparing the activity of enzyme sets at their farthest spacings of 30 nm to the enzyme activity at 5 nm, the spacing effect on activity is nearly 2-fold for the different-strut reaction (overall increase of 60%) versus same-strut (an overall increase of 35%). Such differences may arise due to continuous versus discontinuous structures, which correspond to the same-strut versus different-strut organizations, respectively. This structural difference may provide a more consistent region through which substrate channeling can occur, by limiting diffusion into bulk solution and maintaining a higher local concentration of substrate and intermediate along the scaffold. This conclusion is supported by previous work utilizing a generic protein bridge between glucose oxidase and horseradish peroxidase on a DNA sheet to induce a “bridge-based cascade”, where a continuous hydration layer enabled substrate channeling over an extended distance.28 Particularly over larger distances, where such channeling mechanisms would have a greater effect, discontinuous origami features induced by the different-strut layouts would have a more pronounced detrimental effect, as indicated by our results (Figure 4C).
CONCLUSION

The presented study elucidates design considerations in the rapidly expanding field of enzymatic reactions on engineered scaffolds. The ability to incorporate a model enzyme cascade (GOx and HRP) onto a 3D wireframe reveals the effects of enzyme spacing, arrangement, and scaffold structure and features on cascaded enzyme reactions. As demonstrated in our study, colocalization on the same wireframe origami structure leads to an increase in cascaded activity that predominates over specific enzyme spacings and relative placements beyond the size regime of the proteins themselves. However, we do find that differences in cascade activity between colocalized enzymes do arise at both small spacings and larger spacings. As enzyme spacing becomes smaller, an activity increase occurs until approximately 5 nm, whereas fewer steric limitations on enzyme binding appear to reduce colocalized yields. Activity increases at small spacings have been explained by the overlapping of enzyme hydration layers, effectively allowing direct transfer of intermediate without significant bulk diffusion. A densely packed and highly charged DNA scaffold may serve as a bridge connecting the enzyme environments, and in turn would have a more pronounced effect at larger length scales. In this regime, structural continuity of the scaffold between the enzyme linkages supports low-dimensional substrate diffusion, promoting substrate channeling and thus a consistent and beneficial local environment over which the enzyme reactions proceed.

As applications in enzymatic catalysis grow, and our ability to synthesize and define structure at the nanoscale becomes more precise, it becomes increasingly important to probe how nanoscale environments affect catalytic reactions. Using a library of enzyme layouts on individual DNA wireframe scaffolds, this study confirms that significant benefits to enzyme scaffolding can be achieved with DNA without the need for spacing of components on the order of enzyme dimensions, regardless of the specific mechanisms behind documented enhancements in scientific literature. Furthermore, DNA origami structure considerations and local environment in the enzyme layouts, as opposed to specific DNA sequence makeup at enzyme binding sites, appear to play a more significant role in large-spacing regimes. This opens the ability to further tailor nanoscale environment in cascaded enzyme systems and provides experimental support that mesoscale organizations can yield significant enhancement in activity without direct substrate channeling between enzymes.

METHODS

DNA Origami Synthesis

A solution of 20 nM m13p18 phage, ssDNA scaffold was mixed with a 5x excess of staples (main staple sequences listed in Table S1 with general sequence layout and labeling scheme shown in Figure S8) in 1x TAE buffer containing 12.5 mM MgCl2. The cdNANano file, used to design and visualize the staple layout, is included as Supporting Information. Staples used for enzyme binding onto the selected origami location are replaced by staples possessing the necessary ssDNA overhang with specific replacements detailed in Tables S2–S5 based on the experimental setup. The mixture was then subjected to an annealing procedure, according to previous literature, consisting of a slow cooling from 95 to 20 °C over a period of 14 h. Excess strands were removed by repeated washings (5x) of the solution through 100 kDa spin filters (MilliporeSigma). Final concentrations were measured by UV–vis (Cary-Agilent).

DNA Strand Deprotection

Protected 5′ sulfide-DNA were deprotected by addition of TCEP to yield thiolated binding strands (5′-/3′thioMC6-D/TGGATATAGGTAGTGAAGT-3′ for GOx) and for (5′-/3′thioMC6-D/TGGATATAGGTAGTGAAGT-3′ for HRP). The strands were incubated with TCEP in a 1:100 ratio for 1 h, then purified using G Microspin G-25 Columns. Strands were then immediately used for enzyme functionalization.

Enzyme Functionalization

Glucose oxidase and horseradish peroxidase were prepared into stock solutions of 150 μM using a 50% mixture of PBS and glycerol. Enzymes were incubated with a 20:1 excess of Sulfo-EMCS for 1 h in PBS, pH 7.5, and then spin-purified by centrifugation through a 10 kDa size-exclusion filter (Sigma Millipore). Enzymes were then incubated with different ratios of activated DNA:enzyme (10:1 for GOx and 5:1 for HRP) in PBS, pH 7.5 for 12 h. All enzyme work was undertaken at 4 °C. The ratio of DNA binding to enzyme was measured by UV–vis spectroscopy using relative peak comparisons at 450 nm for GOx and 400 nm for HRP versus 260 nm for DNA. Peak comparisons for pure enzyme are shown in Figure S2, which in combination with enzyme calibration curves shown in Figure S3 were used to calculate both functional enzyme concentration and relative number of DNA per enzyme. DNA:enzyme ratios were optimized to provide an enzyme attachment ratio of approximately 1:1. The DNA-functionalized enzyme stocks were stored in a 50% glycerol/PBS solution at −20 °C.

Enzyme Attachment to Origami

Origami and enzyme were incubated in a 1:1.1 ratio (2 mM concentration) in a 2xTE solution (20 mM Tris, 2 mM EDTA) containing 12.5 mM MgCl2, adjusted to a pH of 7.5. The solution was heated at 37 °C for 5 min, cooled to room temperature over 30 min, and then further cooled to 4 °C over an additional 30 min. The mixture was then incubated for 24 h on a rotator at 4 °C and then used immediately.

Activity Assays

Enzyme cascade activity was measured by the substrate Amplex Red, which is reduced by horseradish peroxidase to the fluorescent reporter Resorufin. Concentrations in experimental setups are detailed in the results and figure captions. One hundred microliters of reactions were measured in a 96-well plate using a Tecan Infinite plate reader with four replicates performed for each sample. The ABTS reaction was tracked through absorbance measurements on the same instrument. Experiments corresponding to Figure S1, Figure S2, and Figure S10 were run on a BioTek Cytation 5 plate reader.

TEM Imaging

DNA origami was placed on a TEM grid and negatively stained with uranyl acetate. Imaging was performed at the Center for Functional Nanomaterials at Brookhaven National Laboratory using a Hitachi 3000K TEM.

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.1c00387.

Materials; impact of DNA functionalization on enzyme activity; calculation of enzyme binding efficiency; TEM imaging of origami; absorbance spectra for determining enzyme-DNA functionalization; individual enzyme activity bound to origami; confirmation of enzyme binding distances; full enzyme spacing data regarding positions and reaction time; DNA sequence data for origami and binding sequences for all arrangements; images describing all spaced enzyme setups (PDF)
caDNAnano file for octahedron design included in separate .json file (ZIP)

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Author Contributions

J.S.K. and O.G. conceived the concept and designed the experiments. J.S.K. and Y.X. conducted the experiments. J.S.K. and O.G. wrote the paper. J.H. aided in optical measurements. O.G. supervised the project. All authors discussed the results and comments on the manuscript.

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Notes

The authors declare no competing financial interest.

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