Signaling Cascades | Hot Paper

Signal Propagation in Multi-Layer DNAzyme Cascades Using Structured Chimeric Substrates**

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Abstract: *Signal propagation through enzyme cascades is a critical component of information processing in cellular systems. Although such systems have potential as biomolecular computing tools, rational design of synthetic protein networks remains infeasible. DNA strands with catalytic activity (DNAzymes) are an attractive alternative, enabling rational cascade design through predictable base-pair hybridization principles. Multi-layered DNAzyme signaling and logic cascades are now reported. Signaling between DNAzymes was achieved using a structured chimeric substrate (SCS) that releases a downstream activator after cleavage by an upstream DNAzyme. The SCS can be activated by various upstream DNAzymes, can be coupled to DNA strand-displacement devices, and is highly resistant to interference from background DNA. This work enables the rational design of synthetic DNAzyme regulatory networks, with potential applications in biomolecular computing, biodetection, and autonomous theranostics.*

Cells use enzymatic signaling pathways for a number of critical functions, including the detection of environmental stimuli, signal amplification, and regulated information propagation through the intracellular environment. Cells typically implement these functions using proteins, but the complexity of protein folding makes the rational design of protein-based signaling cascades infeasible.[1] Although prior work on biocomputing devices using naturally occurring proteins shows promise,[2] this approach is limited by the possible protein–protein interactions. DNA, on the other hand, is an ideal alternative engineering material for the de novo design of synthetic enzymatic cascades, thanks to predictable Watson–Crick base pairing and secondary structure formation. Synthetic analogues of some basic cellular processes have been implemented with DNA, including computation,[3] self-assembly,[4] locomotion,[5] small-molecule sensing,[6] and catalysis.[7] Herein, we focus on DNAzymes[8] (also known as deoxyribozymes), which are single-stranded DNA molecules that can catalyze many of the same reactions as protein enzymes[9] and have been used for computation in parallel gate arrays.[3c, 10] We report a DNAzyme cascade system that uses structured, single-stranded substrates to sequester activating sequences and to propagate an activating signal to a downstream DNAzyme when cleaved by an upstream DNAzyme. We have developed multi-layer signaling cascades and logic circuits, in which a conformational change in a molecule propagates information downstream, mimicking biological systems that rely on modifications such as phosphorylation of downstream enzymes to propagate information.

We based our designs on the most widely used family of DNAzymes: RNA-cleaving DNAzymes. With appropriate metal cation cofactors, these DNAzymes cleave RNA or chimeric DNA/RNA substrates in a multiple-turnover reaction, providing built-in signal amplification capabilities. For a given catalytic motif, DNAzyme–substrate pairs can be designed by simply choosing appropriate complementary sequences for the substrate and the substrate-binding arms of the DNAzyme. This is considerably simpler than designing enzyme–substrate pairs de novo by protein engineering. We used the 8–17 RNA-cleaving DNAzyme because of its compact size and efficient catalytic rate.[11]

This work is built on previous work on ribozyme circuits[12] and on DNAzyme signaling cascades that either sequestered the downstream effector sequence in a partially complementary complex[13] or built two-layer cascades where the downstream DNAzyme generated a colorimetric readout.[14] In the first case, the use of a multi-strand complex as the mediator increases the number of strands and the complexity of circuit preparation. In the second case, the downstream DNAzyme cannot propagate the signal further within the molecular circuit. Although signal amplification has also been demonstrated using DNA strand displacement[7c, 15] and catalytic hairpin assembly,[16] these circuits must be specifically designed to obtain catalysis, for example, using seesaw gates.[3a,b, 17] We use DNAzyme displacement reactions,[18] which combine the advantages of strand displacement to program reaction pathways with the inherent catalytic ability of DNAzymes. This reduces the number of DNA strands needed to achieve signal amplification.

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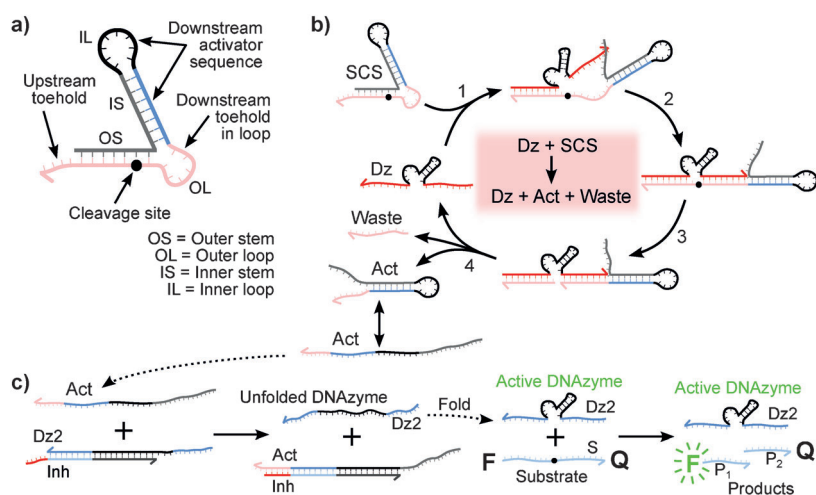
Figure 1. SCS design and mechanisms for SCS cleavage and DNAzyme displacement.
a) Design of a SCS to enable signaling between DNAzymes. The SCS consists of an outer stem and loop, which make up the upstream DNAzyme binding domain (red), and an inner stem and loop, which sequester a downstream activator sequence (blue and black). The cleavage site is located towards the inner end of the outer stem. The gray cage sequence is chosen to fold into the desired structure, producing a topological constraint on the downstream reaction kinetics that is undone when the SCS is cleaved by the upstream DNAzyme. b) Mechanism of cleavage of the SCS by an upstream DNAzyme (Dz). The upstream DNAzyme binds to the outer stem and loop by toehold-mediated strand displacement. The cleavage reaction produces a waste strand and an activator strand (Act). In the activator structure, the outer loop has been released from the topological constraint previously imposed by the outer stem, making the downstream toehold in the outer loop available to bind with a downstream circuit element. c) Mechanism of the DNAzyme displacement reaction. The catalytic activity of the downstream DNAzyme strand (Dz2) is inhibited by hybridization to a partially complementary inhibitor strand (Inh) with a short overhanging toehold. Activation occurs by a toehold-mediated strand-displacement reaction: An input strand (Act) binds to the complex (Dz2–Inh) through the toehold. The input initiates a branch-migration reaction that eventually displaces a catalytically active downstream DNAzyme strand (Dz2), leaving an inert waste complex (Act–Inh). The DNAzyme strand then folds into a catalytically active conformation and proceeds to bind to substrate molecules (S) and cleave them, producing shorter cleavage products (P_1 and P_2). The cleavage reaction causes separation of the fluorophore–quencher pair attached to the two ends of the substrate, which is observed as an increase in bulk fluorescence that is due to a loss of fluorescence resonance energy transfer (FRET).

In cellular enzymatic signaling cascades, an activation signal is typically passed from one enzyme to another through chemical modifications. In this work, information propagation between enzymatic units is achieved through the covalent modification of a structured chimeric substrate (SCS). The SCS uses a metastable dual stem–loop design[19] (Figure 1a) and comprises several domains that make up interchangeable input and output modules. The use of a modular intermediary simplifies the design process by removing the need for direct enzyme–enzyme interactions, which are often found with protein-based cascades, such as phosphorylation in the MAPK pathway.[20] The inner 7 bp stem and 8 bp loop constitute the output module, whose secondary structure weakly sequesters a downstream activator. The outer 7 bp stem and 6 bp loop stabilize the structure and protect the activator toehold in the outer loop, thus preventing unwanted interactions with the downstream DNAzyme before cleavage. The outer stem and loop also constitute the input module, with a substrate-binding and -cleavage domain for an

upstream DNAzyme. We minimized the size of the outer loop to better protect the toehold, which led to a 5 bp overlap between the upstream DNAzyme binding arm and the downstream inhibitor toehold sequences. As shown in Figure 1b, an upstream DNAzyme interacts with the SCS when one of the 8 bp substrate-binding arms hybridizes with the 4 bp outermost toehold and opens the outer stem through a toehold-mediated strand-displacement reaction.[15b] The second arm binds the outer loop, linearizing the substrate domain and correctly positioning the SCS cleavage site opposite the catalytic core of the DNAzyme. The subsequent cleavage reaction causes the outer stem to dissociate as waste, freeing the protected toehold in the outer loop of the SCS, which can now hybridize to its complement more effectively. The relatively weak secondary structure in the activator released by SCS cleavage allows it to interconvert between hairpin and linear structural forms. Thus, downstream interactions are not impeded by the secondary structure of the activator.

This mechanism is particularly suited for use with our previously reported DNAzyme displacement logic gates,[18] in which DNAzyme catalysis is controlled using toehold-mediated DNA strand-displacement reactions.[15b] The activator released by SCS cleavage binds to the toehold of the downstream DNAzyme–inhibitor complex and undergoes branch migration to displace a catalytically active DNAzyme strand, producing an inert waste complex (Figure 1c). The displaced DNAzyme refolds into a catalytically active conformation and can then cleave its own substrate. Thus, activation of one DNAzyme species causes the activation of a second DNAzyme species, implementing signal propagation.

For correct behavior in synthetic multi-enzyme systems, each enzyme must interact with its intended substrate with high specificity. In protein-based enzymatic cascades, specificity is derived from complex interactions between the secondary and tertiary conformations of both enzyme and substrate, rendering rational design of such interactions infeasible. DNAzyme-based cascades achieve specificity through sequence-specific hybridization to substrates. We can modify the SCS input and output modules to enable signaling between DNAzymes with different substrate binding arms while keeping the SCS structure intact. As the SCS does not need to be redesigned for each subsequent layer, this enables the rapid construction of two-, three-, four-, and five-layer linear DNAzyme signaling cascades, each initiated by the addition of active top-layer DNAzymes (Figure 2). Each cascade uses the same reporter layer (layer 1), with layer $n + 1$ added upstream of layer n to extend the cascade. This naming system reflects the sequence commonality between each layer
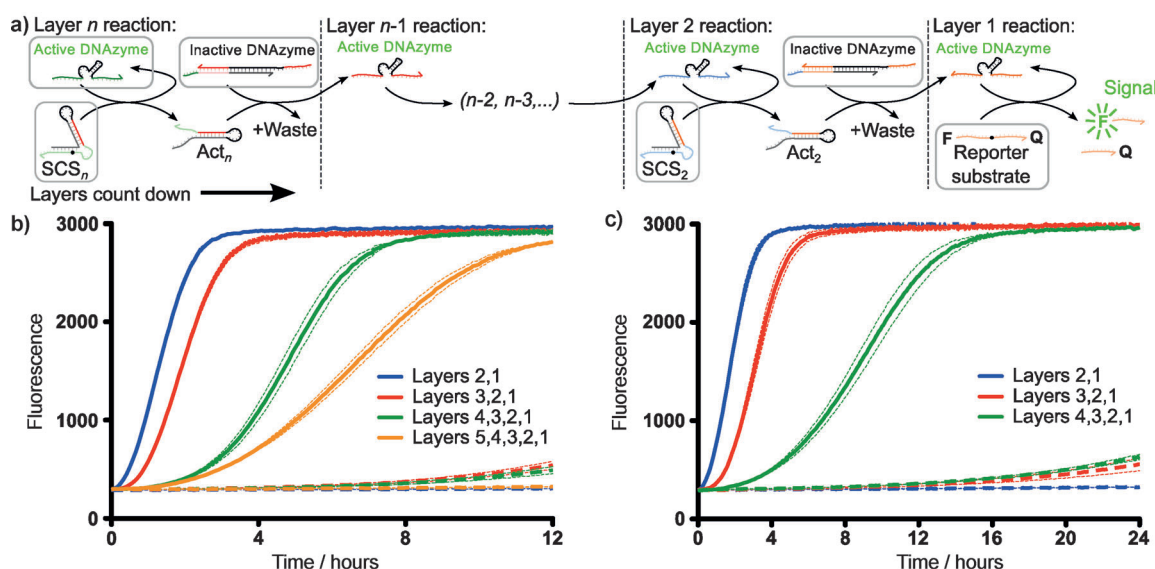
Figure 2. Demonstration of DNAzyme signaling cascades. a) Multi-layer DNAzyme signaling cascades using DNAzyme displacement reactions. Initial species for each layer of the cascade are highlighted in grey boxes. In each layer, an active DNAzyme cleaves the corresponding SCS, producing an activator that releases the downstream DNAzyme from its catalytically inactive enzyme–inhibitor complex through a DNAzyme displacement reaction, thereby propagating the activating signal to the next layer of the cascade. b) The mean fluorescence signal (solid lines) from two-layer (blue), three-layer (red), four-layer (green), and five-layer (orange) linear DNAzyme signaling cascades with equimolar (100 nM) DNAzyme concentrations in each layer. The dashed line represents the same reaction without the top-layer active DNAzyme, which measures the non-specific activation (leakage) of the downstream circuit. The dotted lines represent the 95% confidence interval from three replicate experiments. c) Kinetic traces for multi-layer linear DNAzyme signaling cascades with increasing DNAzyme concentrations in each layer (25 nM in layer 4, 50 nM in layer 3, 75 nM in layer 2, and 100 nM in layer 1) to demonstrate signal amplification. In both plots, dotted lines represent the 95% confidence interval from three replicate experiments.

n, irrespective of the cascade length. Our five-layer cascade is the longest DNAzyme signaling cascade implemented to date. The development of extended catalytic signaling cascades with a high signal-to-noise ratio is challenging because an unwanted signal that is generated in the absence of input (leakage) is also amplified by downstream circuit elements. Kinetic traces of multi-layer cascades (Figure 2b) show that the time taken for cascade execution increases with the number of layers (Supporting Information, Section S1). Lower DNAzyme concentrations reduce leakage at the expense of activation speed by relying on multiple-turnover cleavage for signal amplification (Figure S1). In particular, using lower concentrations in the upstream layers of the cascade with increasing concentrations in each downstream layer can reduce leakage without affecting the maximum output level or a significant sacrifice in speed (Figure 2c). Additional controls using uncleavable SCS molecules demonstrate that cleavage is necessary for signal propagation (Figure S2). Therefore, we have demonstrated that chemical modification of a structured substrate by a DNAzyme can be used to propagate information in a signaling cascade.

As DNA interactions are sequence-specific, the SCS can interact with any upstream or downstream circuit components with the correct sequence. We implemented signaling cascades between a variety of DNA logic components in both the upstream and downstream positions, including various DNAzyme logic gates and a strand displacement reporter gate (Figure S3). This demonstrates the flexibility of DNAzyme-based interactions through the SCS, which enables the development of hybrid DNA circuits that comprise compo-

nents from multiple architectures, which is currently a significant challenge.

Multi-layer synthetic DNAzyme logic cascades offer a route to increasing the sophistication of biomolecular logic circuits, with the long-term aim of enabling the robust isothermal detection of disease states through sequence-specific nucleic acid detection[14,21] or aptamer-based detection of small molecules.[6a–c] Incorporating logic into enzymatic cascades enables the integration of multiple input signals, which can reduce false positives in bioassays and enable the detection of disease states where a single target is insufficient for an accurate diagnosis. To illustrate the potential of DNAzyme logic cascades for detecting multiple pathogenic targets in extracted DNA, we implemented multi-layer circuits for typing representative pathogen signatures from all four dengue virus serotypes (DEN1–4). Dengue is a major global health concern,[22] and accurate serotyping is important because sequential infection with different serotypes is a risk factor for dengue hemorrhagic fever and dengue shock syndrome, both of which can be fatal.[23]

We exploited the modularity of the SCS to design a two-layer, three-input AND circuit template, in which two DNA oligomers derived from conserved sequences within the ssRNA dengue genomes and a serotype-specific DNA oligomer must be present to produce a fluorescence output. As shown in Figure 3a, each layer of the circuit is an AND gate activated by two inputs in a cooperative displacement reaction.[24] The use of mismatches in the inhibitor is required for rapid release of the DNAzyme, because the catalytic core is not displaced by either input.[18] One of the inputs of the
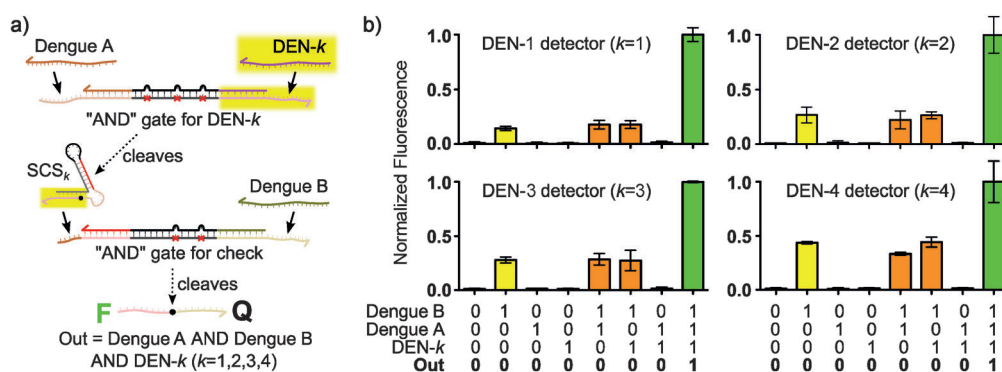
Figure 3. Exemplary application of the SCS in a multi-layer diagnostic logic circuit. a) Design of multi-layer diagnostic logic circuits for the detection of sequences from the genomes of all four dengue serotypes. The circuit template for serotype DEN-k ($k = 1, 2, 3, 4$) requires the presence of two conserved sequences from the dengue viral genome (Dengue A and Dengue B) and one sequence specific to the serotype of interest (DEN-k). This is implemented by DNAzyme displacement AND gates with mismatched inhibitors,[18] which are connected by a SCS molecule. When both upstream inputs are present, the active upstream DNAzyme cleaves the SCS, producing an activator that serves as one input to the downstream gate. If the second input to the downstream gate is also present, the downstream DNAzyme will be activated, which is observed by a loss of FRET following substrate cleavage. The upstream AND gate uses the three-mismatch design, characterized in our previous work,[18] whereas the downstream gate uses an asymmetric pattern of mismatches because the activator produced by the SCS only partially displaces the catalytic core. We derived detection circuits for all four dengue serotypes (DEN-1–4) by modifying only the domains highlighted in yellow. b) Serotyping circuits for DEN-1–4, showing the correct operation of all four instantiations of the three-input AND circuit template. Each serotyping circuit was characterized using all eight combinations of the two conserved sequences and the correct serotype-specific sequence. Variations in the normalized fluorescence levels (i.e., different levels of activation and leakage) may be attributed to variations in the stability of the corresponding SCS$_k$ structures in each case. Error bars represent the 95% confidence interval from three replicate experiments.

the functionalities of cellular cascades: integration of multiple input signals, signal amplification, transduction, and propagation. The combination of DNAzymes, strand displacement, and rationally designed structured chimeric substrates enabled us to implement synthetic signaling cascades that are compatible with a variety of DNA logic gates, including the longest DNAzyme signaling cascade demonstrated to date. These DNAzyme cascades hold promise for practical applications, such as pathogen detection. We illustrated this by demonstrating that our circuits resist background interference and can implement multi-input, multi-layer detection of multiple pathogen signatures.

Future work will explore the operation of DNAzyme cascades in physiologically relevant conditions,[25] such as cell lysate[26] or serum, which may be challenging because of the presence of nucleases that may degrade circuit components, or because of insufficient concentrations of the metal ion cofactors that are required for efficient DNAzyme catalysis.[27] Furthermore, the modular design of the SCS should allow the implementation of increasingly complex synthetic DNAzyme signaling networks, incorporating network motifs such as feedforward and feedback cycles.[28] These circuits could exhibit non-trivial dynamic behavior to enable more sophisticated decision-making for diagnostic and therapeutic applications, possibly connected to alternative readout technologies such as gold nanoparticles[29] or paperfluidic devices.[21a, 30]

downstream gate is released upon cleavage of the SCS. We replicated this template, modifying the highlighted parts of the upstream AND gate and the SCS, to produce four circuits, each sensitive to a different serotype-specific target sequence (Figure S4). We observed strong positive responses from all four circuits in the presence of all three signatures; these responses were at least 2.5 times stronger than the maximum response seen in the absence of one or more signatures (Figure 3b). Higher leakage is seen in the presence of the downstream Dengue B input, suggesting that there is some interaction between the SCS and the downstream AND gate prior to SCS cleavage. Misfolding of SCS or enzyme strands reduced the system performance (Figure S5), showing that optimization of the predicted secondary structure is important for efficient circuit operation.

To test our approach in a minimal biological background, we implemented a two-layer DNAzyme cascade using the SCS with increasing amounts of random background DNA (Figure S6). This models a common detection scenario in which all the nucleic acids have been extracted from a sample for analysis. We showed that the SCS design is sufficient for this minimal assay detection environment. Furthermore, all experiments described herein were performed using minimal oligonucleotide purification techniques, which is essential for the development and use of low-cost bioassays. Therefore, we have demonstrated two key properties for a practical bioassay: robust operation in background and straightforward preparation.

In summary, we have developed a method to design extended DNAzyme signaling cascades that exhibit many of

Experimental Section

Materials: All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotide sequences are listed in the Supporting Information, Tables S1–8. DNAzymes and inhibitors were purchased with standard desalting whenever possible, with the exception of oligonucleotides that exceeded 60 base pairs in length (which were PAGE purified by the manufacturer, in accordance with the manufacturer's recommended procedures). All DNA/RNA chimeric substrates (SCS molecules and fluorescent reporter substrates) were purified by RNase-free HPLC by the manufacturer. The fluorescent reporter substrates were labeled with a 5′-FAM quenched by a 3′-TAMRA fluorophore. Oligonucleotides were resuspended in RNase-free H$_2$O (Sigma–Aldrich) in accordance with the specifications provided by the manufacturer at a stock

concentration of 50 μM. Working stocks were made by adding 50 μL of the resuspended oligonucleotide solution into 950 μL buffer.

Preparation of DNAzyme–inhibitor complexes and SCS molecules: DNAzyme strands and inhibitor strands were pre-complexed by heating the DNAzyme and inhibitor strands together at 95 °C for 3 min on a heat block, and subsequently annealing by cooling to room temperature over a minimum of 90 min. In many cases, an excess of inhibitor relative to DNAzyme was used, to ensure complete inhibition of the DNAzymes—in these cases, the resulting solution of DNAzyme–inhibitor complexes and excess free inhibitor strands was used without further purification. Single-stranded SCS molecules (and loop-inhibited DNAzymes) were prepared using the same heating and annealing method.

Assay conditions and instrumentation: All assays were performed at room temperature (23 °C) in a buffer of NaCl (1M), HEPES (50 mM), ZnCl$_2$ (1 mM) at pH 7.0. Fluorescence was read either on a Quantamaster 40 fluorimeter (PTI, Binghamton, NJ) in a 300 μL reaction volume or on a Spectramax M2e fluorescent plate reader (Molecular Devices, Sunnyvale, CA) in a 200 μL reaction volume. In all cases, fluorescein emission was monitored at an excitation wavelength of 492 nm and an emission wavelength of 518 nm. Error bars indicate two standard deviations from the mean of three replicates, representing the 95% confidence interval. Full details of the assay conditions for individual experiments are given in the Supporting Information.

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