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Communication

Using Strand Displacing Polymerase To Program Chemical Reaction Networks

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ABSTRACT: Chemical reaction networks (CRNs) provide a powerful abstraction to formally represent complex biochemical processes. DNA provides a promising substrate to implement the abstract representation (or programming language) of CRNs due to its programmable nature. Prior works that used DNA to implement CRNs either used DNA-only systems or multienzyme DNA circuits. Architectures with DNA-only components had the rationale of being biologically simple systems. Multienzyme systems, on the other hand, aimed at using natural enzymes to improve circuit performance, although, at the cost of increased complexity. In this work, we explore an alternative architecture that lies along the spectrum in between DNA-only systems and multienzyme DNA systems. Our architecture relies on only a strand displacing polymerase enzyme and DNA hybridization reactions for implementing CRNs. First, we briefly introduce the theory and DNA design of simple CRNs and then explore the fundamental properties of polymerase-based strand displacement systems. Finally, we engineer a catalytic amplifier *in vitro* as a use-case of our framework since such amplifiers require the intricate design of DNA sequences and reaction conditions.

Nature uses sophisticated biochemical networks for cellular and molecular regulation. For example, the biochemical sensors in our body produce melanin to protect us from harmful UV rays.[1] Another example would be the circadian rhythm of our body, which clocks our daily time to sleep, eat, and work.[2] Therefore, the ability to design programmable biochemical controllers synthetically is essential since such biochemical networks are what governs life. A synthetic biocontroller, for example, programmed to regulate the cortisol release could help prevent the flattening of the diurnal cortisol curves,[3] as flatter slopes are commonly associated with poor mental health.

Chemical reaction networks (CRNs)[4−7] model the dynamics of the species in a well-mixed solution. Their computational power has been extensively studied in the past few decades, and they are long known to be able to compute complex functions.[8−10] Therefore, CRNs provide an ideal abstraction for designing complex biochemical networks synthetically. However, such use of CRNs as models of computation, and therefore a programming language for biology, is contrary to the traditional use of CRNs that simply modeled and evaluated the mass-action kinetics of biochemical systems. Prior to the early DNA design for implementing CRNs,[7] this issue mainly stemmed from the lack of a tunable biochemical substrate that can simulate arbitrary chemical kinetics.

DNA nanotechnology offers a suitable solution to this problem since as a substrate DNA is biocompatible and highly programmable.[4−6,11−18] In fact, there have been prior architectures to engineer biochemical networks synthetically using only DNA,[4,6,7] as well as biologically complex multienzyme architectures.[19,20] The rationale for enzyme-free DNA systems is to engineer biologically simpler systems and do all the programming with Watson−Crick base pairing. Multienzyme systems such as the PEN DNA toolbox[21] instead uses polymerase, exonuclease, and nicking enzyme to achieve dynamic behaviors. Both systems have demonstrated substantial progress at engineering complex biochemical systems;[4,6,20] however, their designs possess several challenges. For example, the enzyme-free Displacillator architecture is fairly complex and not nearly as efficient as polymerase chain reaction (PCR) at exponential amplification.[6] Enzymatic systems such as the PEN toolbox have a compact architectural design; however, they require careful control of reaction conditions since several enzymatic components are involved.[21] For example, besides environmental and buffer conditions, the use of a slower nicking enzyme places restrictions on the DNA sequence around the enzyme's recognition site.[19]

In this work, inspired by the phenomenal progress of DNA-based designs that implement CRNs,[4,6,19,21] we propose a novel way to implement CRNs (refer to Figure 1 and Figure S1). We only use a strand displacing polymerase enzyme, originally proposed in theoretical work,[5] to implement CRNs. Using a polymerase-based design is a natural extension to DNA-only systems since the polymerase enzyme provides a fast and powerful source of energy by the hydrolysis of dNTPs. Theoretically, it has been shown that the polymerase architecture can implement arbitrary CRNs;[5] however, this work is the first to carefully study the properties of polymerase-based chemical systems experimentally. We explore several important fundamentals of polymerase-based strand displace-

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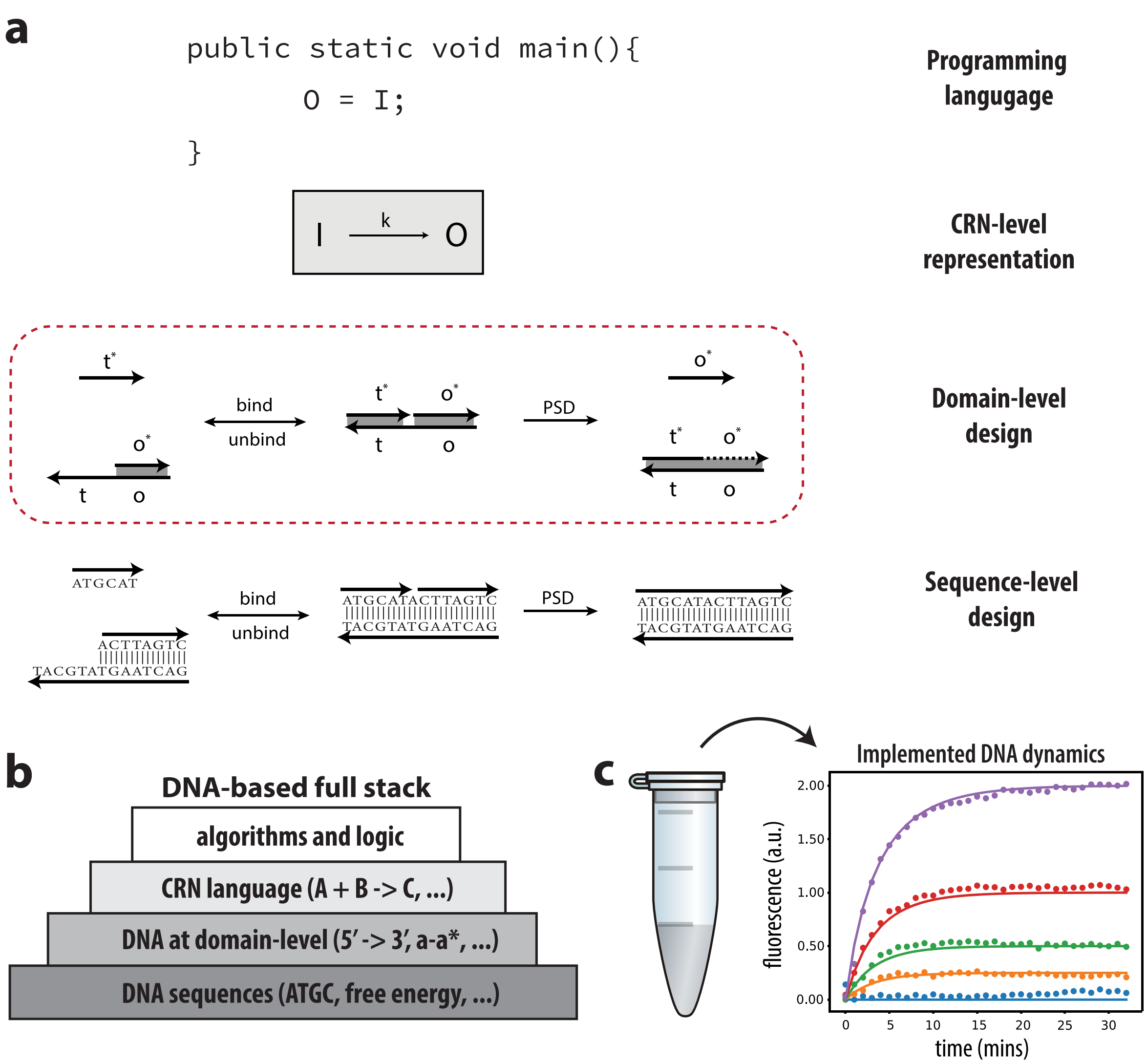

Figure 1. DNA computing full stack. (a) At the highest level, a C-like general purpose programming language is expressed using a set of CRNs. This intermediate representation gets translated to domain-level DNA design and then implemented using a set of DNA strands. (b) Full stack showing the parameters of focus and representation. (c) The implemented DNA system is visualized using fluorescence spectroscopy. The fluorescence curve is for illustration purposes only.

ment such as tunable rate parameters,[22] range of programmable rates,[23] and mismatched primer-template sequence[24,25] to stop the polymerase activity. As a proof-of-principle, we engineer a catalytic amplifier since such amplifiers require careful design of DNA sequence and also serve as a useful class of sustainable biochemical systems.

Prior work on DNA computing has already demonstrated how polymerase-based circuits can be faster and more compact.[14] However, since they implement binary logic operations, their system does not allow programming of reaction rates and is not catalytic. Moreover, one of the benefits of their fast and compact architecture is the use of ssDNA gates; however, such design limits multioutput DNA complexes and therefore does not utilize the full analog spectrum offered by biochemical systems.

The strand displacement mechanism using a DNA polymerase is slightly different from the toehold mediated strand displacement.[18] There is no tug-of-war between the input and the incumbent strand in polymerase-based strand displacement (PSD) (refer to Figure S2). Once the primer strand hybridizes to the double-stranded gate complex and a polymerase starts priming, it is an irreversible process.

The first step toward the implementation of arbitrary CRNs includes reporter calibration and polymerase activity tests since the polymerase enzyme usually does not operate at room temperature. To do so, we designed a dsDNA reporter complex with a fluorescent dye and a quencher. The complex contains a small exposed region on the 5′ end for the primer strand to attach (refer to Figure S3). Upon hybridization of the primer strand, the polymerase enzyme should displace the fluorescent strand away from the quencher and lead to fluorescent activity (refer to Figure S3a). We tested several input concentrations for a given concentration of reporter complex, as shown in Figure S3b. This simple test confirmed (a) the strand displacement activity of Bst 3.0 polymerase at 50 °C, (b) the output fluorescence is limited by the input concentration, and (c) a linear separation between normalized steady-state fluorescence. Therefore, we can use the reporter concentration to normalize fluorescence curves.

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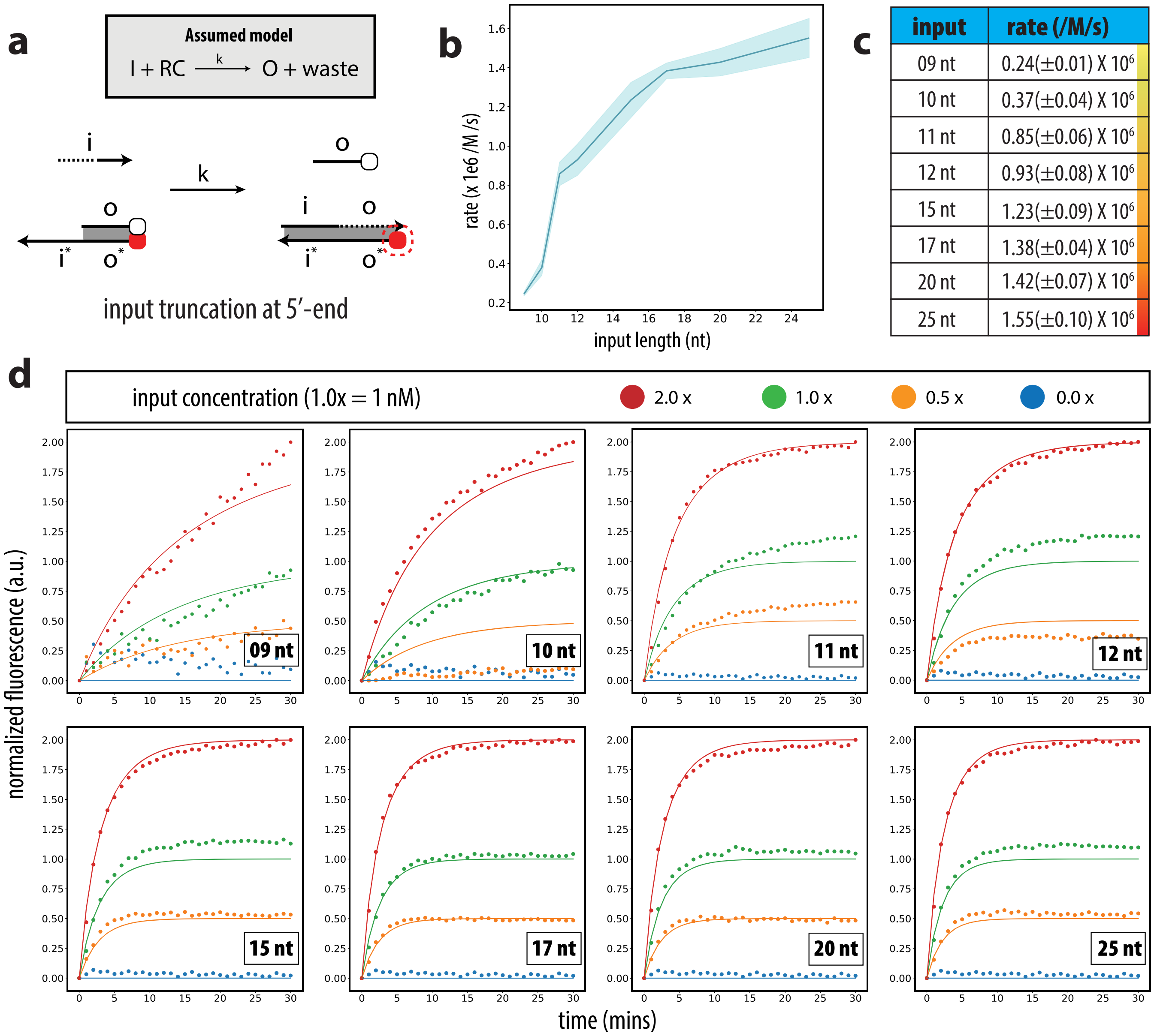

Figure 2. Fundamental tests for evaluating the programmable nature of a PSD system. (a) A single-step model and its DNA implementation to verify stoichiometry and reaction rate. (b−d) The input length is tuned from 9 nt to 25 nt, and its concentration is varied from 0.0× to 2.0×. The model fits and the estimated reaction rates are summarized in a graphical and tabular fashion.

It should be noted that the careful handling of the input strand is required during fluorescence experiments. This is because inputs are added at room temperature while the reaction master mix operates at 50 °C (refer to Figure S4). If too much time is taken to add input to the plate reader, the plate reader will disperse the incubated heat and this can affect the reaction kinetics since the plate reader takes a few minutes to restore temperature set point.

For implementing a CRN, the output and input stoichiometry should match and the rate of reaction should be programmable. Therefore, we tested the two fundamental properties of a CRNs: (a) stoichiometry and (b) reaction rate. To control the stoichiometry and tune the rate, we tested the most basic reaction network, i.e. I $\xrightarrow{k}$ O (refer to Figure 2). We changed the length of input (or primer) strand from 9 to 25 nt and also tested several input concentrations from 0.0× to 2.0×, where 1.0× = 1 nM. Using these experimental curves, we fit a single-step model I + RC $\xrightarrow{k}$ O + waste to estimate the values of rate constant k. The estimated rate constants are shown in Figure 2b and Figure 2c. These results demonstrate (a) the rate of PSD is configurable, (b) the reaction rates can be tuned roughly an order of magnitude apart, and (c) the simple single-step reaction adheres to the input−output stoichiometry.

The design for implementing arbitrary unimolecular and bimolecular CRNs is a two-step[5] process (refer to Supporting Information Section 3). The input strand A produces intermediate product I upon PSD, which then releases output strand B. The output activity is reported using a fluorescent complex (refer to Figure 3a). The fluorescence data of the implemented three-step noncatalytic reaction A $\xrightarrow{k}$ B is shown in Figure 3b. The left subgraph keeps the input length constant at 30 nt (varied input concentration) while the right subgraph keeps the input concentration constant at 6× (varied input

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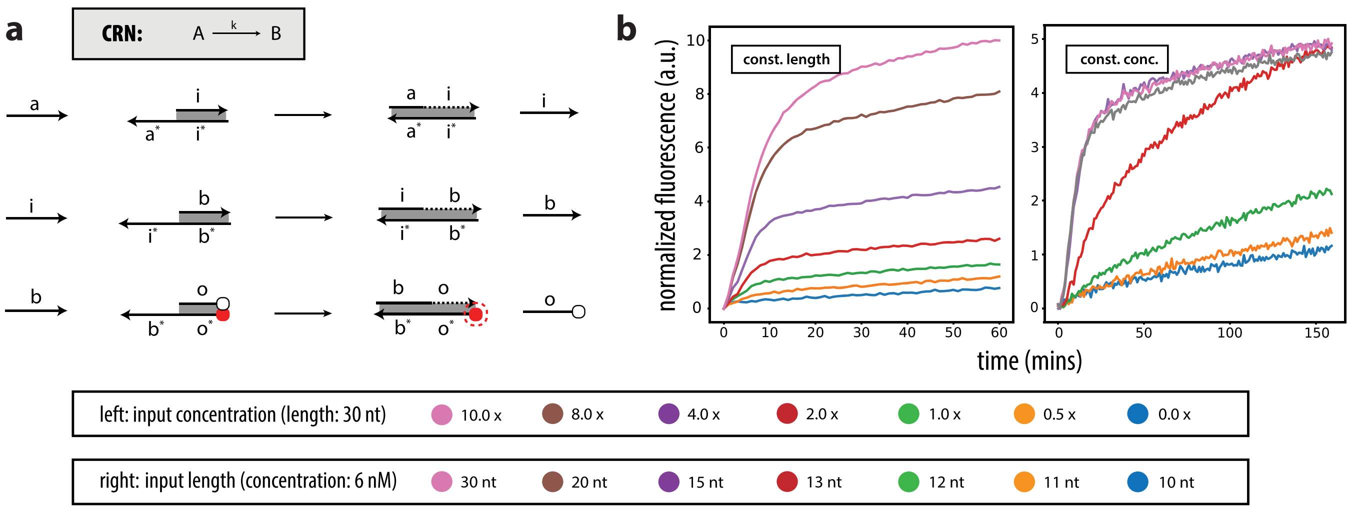

Figure 3. Single-output noncatalytic CRN. (a) The three-step process that implements A $\xrightarrow{k}$ B. (b) The fluorescence data for the implemented CRN at different input concentrations and lengths.

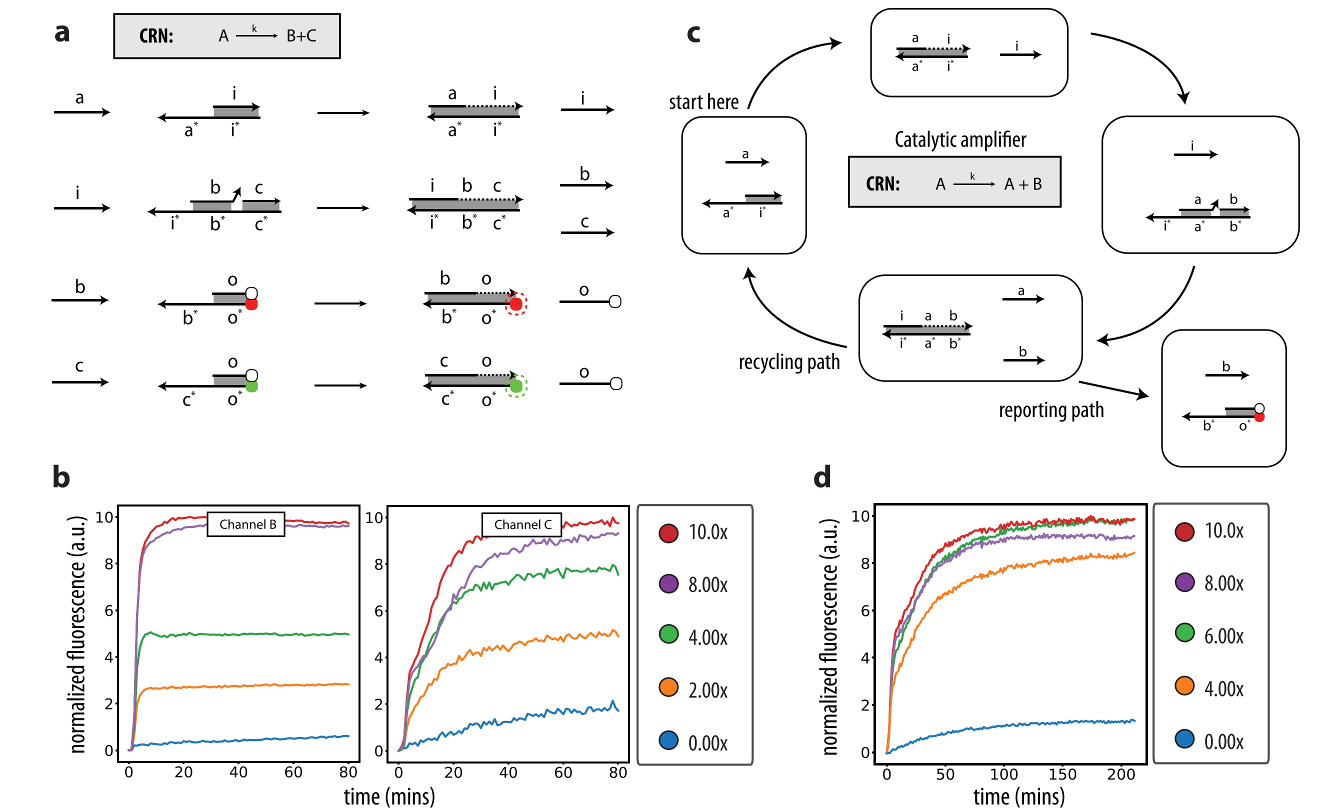

Figure 4. Multioutput CRNs. (a−b) The three-step process that implements A $\xrightarrow{k}$ B + C and its fluorescence data. A slightly lower signal-to-noise ratio is observed in channel C as compared to channel B since the overhang cannot completely stop the polymerase activity. (c−d) The three-step process that implements A $\xrightarrow{k}$ B + C and its fluorescence data. Input A is used as a fuel, and output B is used for reporting.

length). All the partially dsDNA gates were kept at 10× for the left subgraph and 5× for the right subgraph, where 1.0× = 1 nM.

Some output signal activation was observed during the sample incubation phase before adding any input (refer to Figure S5). We conjectured that output signal activity stems mainly due to heavy breathing activity, on both ends, of dsDNA complexes at high temperatures.[6] To test our theory, we added several clamp domains (5−8 nt long) at the 5′ ends of partially double-stranded downstream complexes, shown in green in Figure S5a. The nontruncated fluorescence data before and after clamping is shown in Figure S5c. As seen in the figure, the initial output signal activity is greatly reduced after introducing the clamping thereby confirming our theory of excessive breathing. Therefore, for all further designs, we use 5′ clamping of 5−10 nt to avoid such activity.

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Having verified a single-output CRN, we next tried a multioutput system $A \xrightarrow{k} B + C$ since most systems require the release of multiple outputs. Therefore, we modified the complex in the second step to release two outputs, as shown in Figure 4a. We introduced a 3′ overhang (or primer-template mismatches) to block the polymerase from docking between the two outputs and triggering the gate without the input.[24−26] Since the amplification results are sequence-dependent, we tried a few different mismatches such as poly-T, poly-G, and random N-mer. Based on the fluorescence data (refer to Figure S6b), the poly-T and poly-G chain effectively stop the polymerase activity. Therefore, we use such poly-T overhangs for multioutput noncatalytic and catalytic reactions. The idea of 3′ mismatching is unique and interesting since it can stop polymerase activity in annealed complexes but does not do so when the incumbent strands are released which then acts as a primer for downstream gates. Such dual activity is not possible with other stoppers such as stopper sequences, chemical modifications, etc.[12] which permanently stops the polymerase activity for a given sequence.

After verifying the role of 3′ overhang in inhibiting polymerase, we implemented the multioutput CRN using the three-step process and reported both outputs using two different fluorescence channels. The design and data are shown in Figure 4a, b. All the partially dsDNA gates were kept at 10× while the input was varied from 0× to 10×, where 1× = 1 nM. Note the higher signal leak in channel C as compared to channel B. This is because while the primer-template mismatch inhibits the polymerase from priming, it cannot completely stop it.

Having verified single- and multioutput noncatalytic CRN systems, a natural extension is the demonstration of a catalytic amplifier. Such sustainable amplifiers form an interesting class of tools for biochemists since the input strand is recycled with the help of a supporting catalyst.[27] This means that even at lower input concentrations, as long as it is sufficiently recycled, such systems should activate all of the output.

The design of our catalytic amplifier is similar to the noncatalytic reaction; however, the multioutput complex now releases fuel back to the solution along with the output, as shown in Figure 4c. The input strand combines with the first auxiliary gate AI to release I, which can combine with the output gate to release input A and output B. This input acts as a fuel for other gates while the output combines with a fluorescence reporter. All the gates, including the reporter complex, were kept constant at 10.0× concentration while the input was varied from 0.0× to 10.0×.

Figure 4d shows that due to the catalytic activity of the in vitro amplifier, the output is not limited by the input concentration. However, it should be noted that (a) there is some activity without input signal (or leak reaction), and (b) catalytic amplifier is not completely recycling the input (or fuel loss). The leak reactions are well-known[6] and they mainly stem from DNA synthesis errors, DNA polymerase activity, and improperly annealed gates. However, this activity is much lower than the signal and therefore can be thresholded[6] or avoided completely using newly proposed leakless protocols and principles.[28,29] The input recycling is not ideal since some gates absorb inputs but never release them back to the solution due to inefficient polymerase activity, and poorly annealed gates. Therefore, the catalytic limit of our *in vitro* amplifier lies around 4x input. Finally, it should be noted that while a full steady state convergence at 10x input requires close to 100 minutes, the half-time completion[18] i.e. the time required to activate 5x output fluorescence is only about 20 minutes demonstrating the high speed of PSD reactions.

In this work, we introduced a new architecture that uses a strand displacing polymerase enzyme for implementing chemical reaction networks. First, we demonstrate the concentration and the length of the primer strand as tunable parameters for the stoichiometry and the rate of reaction. Second, we use these principles to implement both noncatalytic and catalytic reactions. The experimental demonstration of a catalytic amplifier serves as a proof-of-principle of our polymerase-based architecture since it requires careful sequence design and systematic implementation. Such experimental demonstrations show that there are alternative avenues of DNA computing and reaction networks yet to be explored.

Our polymerase-based architecture is inspired by the well-known exponential strand displacement amplification (SDA) technique.[30] However, the original SDA design and its newer variants such as PEN toolbox[21] rely on the slower activity of the nicking enzyme to introduce a cut, which can be displaced using a polymerase. Our design successfully eliminated the use of the nicking enzyme making the amplification process simpler, modular, and faster. However, it seems that several open problems still exist. (1) Our design still suffers from the issue of reaction leak, which primarily stems from DNA synthesis errors, improperly annealed gates, and the polymerase enzyme. While the clamping strategy introduced here successfully reduces it, it may not be sufficient for exponential amplifiers. (2) The theoretical requirement of excess auxiliary gate concentration[5] is not very practical during *in vitro* implementations. Even though the excessive concentration of buffered gates[5] can extend the life of the CRN approximations, it does not completely solve the problem. (3) Although polymerase systems are fast,[14] operating at higher temperatures might not always be feasible since it increases reaction leak (excessive breathing) and synthesis cost (longer domains). Therefore, the activity of other enzymes such as Bsu and Phi that operate at lower temperatures needs to be explored. (4) Finally, most complex networks such as oscillators[6] and nanowalkers[31] require careful control of several autocatalytic reactions. Demonstrations of such bimolecular autocatalytic reactions using our framework and solutions to the other mentioned issues remain as open research questions yet to be explored.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c02240.

Materials and methods; control experiments; DNA sequences; temperature dependence of enzyme; clamping activity; discussion (PDF)

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Notes

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