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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Remote Toehold: A Mechanism for Flexible Control of DNA Hybridization Kinetics

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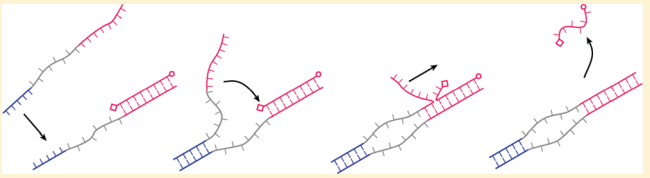
Supporting Information

ABSTRACT: Hybridization of DNA strands can be used to build molecular devices, and control of the kinetics of DNA hybridization is a crucial element in the design and construction of functional and autonomous devices. Toehold-mediated strand displacement has proved to be a powerful mechanism that allows programmable control of DNA hybridization. So far, attempts to control hybridization kinetics have mainly focused on the length and binding strength of toehold sequences. Here we show that insertion of a spacer between the toehold and displacement domains provides additional control: modulation of the nature and length of the spacer can be used to control strand-displacement rates over at least 3 orders of magnitude. We apply this mechanism to operate displacement reactions in potentially useful kinetic regimes: the kinetic proofreading and concentration-robust regimes.



1. INTRODUCTION

DNA is an ideal construction material for self-assembly of nanometer-scale structures because the strength and specificity of interactions can be encoded in the nucleotide sequences of the component strands.[1,2] The operation of dynamic devices such as logic circuits, motors, catalytic networks, and switches requires bonds between strands to be both made and broken. Spontaneous dissociation of double-stranded DNA is slow,[3] so DNA devices typically use strand-exchange reactions in which an invading strand displaces a target strand from a double-stranded substrate.[4]

The rates of strand exchange reactions can be increased 10^6-fold by using toehold-mediated strand displacement.[4−6] Hybridization of the invading strand is initiated at a short single-stranded "toehold" domain attached to one end of the substrate, leading to a branch migration reaction that displaces the target strand from the substrate. In the implementation demonstrated by Yurke and co-workers[4,5] and now widely adopted,[7−21] the toehold and displacement domains are adjacent to each other with no intervening spacer: we refer to this simple architecture as "proximal". A proximal toehold functions both as an address tag and as a means to control the strand-displacement rate and equilibrium. Designs incorporating proximal toehold-mediated strand displacement are limited by the fact that the thermodynamics and kinetics of the reaction are tightly coupled: the binding free energy of the toehold domain determines both the displacement rate and equilibrium concentrations.[5,6] The reaction rate can be tuned by modulating the binding strength of the toehold domain, but, since the rate varies roughly exponentially with the binding strength,[5,6] fine adjustment is difficult. The reaction rate saturates at a toehold length of approximately 6−10 nucleotides (nt),[5,6] which limits the possibility of scaling up DNA networks that use toehold domains as address tags.[8,13,21] Here we introduce "remote" toeholds where toehold and displacement domains are separated by a spacer (Figure 1). We explore the effects of the spacer length and nature (single-stranded, double-stranded, or polyethylene glycol) on the reaction kinetics. We show that reaction rates can be both coarsely and finely tuned and controlled dynamically through control of the properties of the spacer domain. By designing remote-toehold reactions to operate in different kinetic regimes, we show how to improve the sequence discrimination and robustness to concentration variations of strand-displacement reactions.

2. EXPERIMENTAL METHODS

2.1. DNA Design, Synthesis, and Purification. The domain sequences used in this paper were designed to be minimally interacting. Special emphasis was placed on ensuring that single-stranded reactants and products possessed no significant secondary structure, which is known to slow the rates of hybridization and branch migration.[22] Predicted interactions of DNA strands were examined using Nupack[23] and Mfold.[24] The nucleotide sequences of all strands are provided in the Supporting Information. DNA was purchased from Integrated DNA Technologies. Target strands were labeled with 5′-FAM and 3′-Iowa Black and purified by HPLC; all other strands were used without further purification. Concentrations were determined by measurement of the absorbance at 260 nm.

Received: August 14, 2010

dx.doi.org/10.1021/ja1073239 | J. Am. Chem. Soc. XXXX, XXX, 000–000

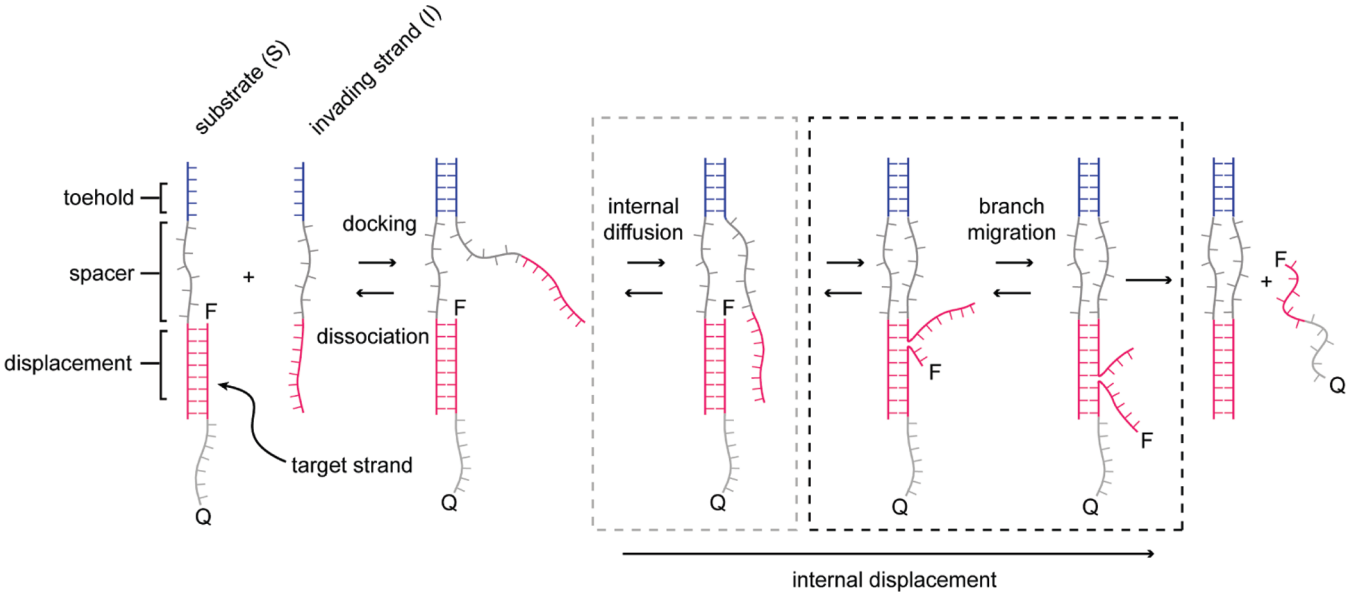


Figure 1. Mechanism proposed for the remote toehold. The target strand, labeled with fluorophore (F) and quencher (Q), is displaced from the substrate (S) by an invading strand (I). The toehold and displacement domains on both the substrate and invading strands are separated by spacer domains. Docking of the substrate and invading strand by hybridization of the toehold domains is followed by an internal diffusion step, which is required to align the displacement domains and initiate the branch migration reaction by which the target strand is displaced from the substrate. Displacement is reported by quenching of fluorescence from the target.

2.2. Spectrofluorimetry. Fluorescence measurements were performed with a Fluoromax-3 JY Horiba fluorometer, at 28 °C in 1.5 mL quartz cuvettes containing a buffer of 10 mM Tris−acetate, 1 mM EDTA, and 12 mM $MgCl_2$. Fluorescence was recorded with excitation/emission wavelengths of 494 nm/520 nm, a slit width of 4 nm, and an integration time of 2 s (Figure 2) or 1 s for all other experiments. Fluorescence traces in all figures were normalized by dividing by the initial signal: with this normalization, a background signal of 0.2−0.3 corresponds to complete displacement of target strands. In all graphs, time $t = 0$ indicates the time of the first measurement after all the initial DNA strands were added to the solutions. The solutions were manually mixed with a pipet set to a volume of 0.75 mL. Adding reagents and mixing the solutions took about 30 s.

3. RESULTS AND DISCUSSION

Our description of the reaction mechanism for remote-toehold strand displacement follows the mechanism described for proximal toeholds by Zhang and Winfree,[6] with the addition of an "internal diffusion" step. The dominant reaction path is sequential (Figure 1). First, the invading strand (I) docks to the substrate (S) through hybridization of its toehold domain. Docking is a second-order reaction. The displacement domain then explores the volume surrounding the docking site (internal diffusion) until it initiates displacement of the target from the substrate: initiation of strand displacement may proceed through hybridization to a short region of spontaneously melted base pairs at the end of the target duplex.[25] Complete invasion of the displacement domain, leading to displacement of the target strand, then proceeds through a branch migration process. Internal diffusion and branch migration are not easily separated experimentally: we encompass them both in a single, first-order internal displacement step. All steps in this sequence are reversible: the invading strand may dissociate from the toehold before it begins to displace the target and may itself be displaced from the displacement domain before the target is removed. However, the additional stability conferred by hybridization to the toehold domain ensures that the probability of return of a target strand, once displaced, is very low.

3.1. Control of Internal Displacement by Modulating the Length and Nature of the Spacer. The relationship between the speed of internal displacement and the nature and length of the spacers was investigated by inserting spacers between the toehold and displacement domains of both the substrate and invading strands (Figures 2 and 3). A high concentration of invading strands and long (14 nt) toeholds were used: under these experimental conditions, the docking step is fast and effectively irreversible, internal displacement is the rate-limiting step, and the overall displacement kinetics are approximately first order. Since in numerous applications of strand displacement the target strand carries additional information in a dangling ssDNA end,[9,13,20,26] the target strand employed here was given a dummy 15 nt ssDNA dangling end. For the sake of clarity, the dangling end is not drawn in Figures 2−4. The 37 nt target strand was synthesized with a FAM fluorophore and an Iowa Black quencher at either end. When hybridized to the substrate, the fluorophore and quencher are separated by 22 base pair (bp) dsDNA and 15 nt ssDNA (approximately 8 nm). When displaced, the target, now single-stranded, assumes a random coil configuration in which the separation between the fluorophore and quencher is considerably reduced, leading to efficient Förster resonant energy transfer (FRET) from the donor to the acceptor[27] and thus reducing donor emission. The progress of the strand-displacement reaction can therefore be observed by measuring the donor fluorescence intensity. In this paper, ss(n)−ds(m) denotes a system comprising an n nt ssDNA spacer in the substrate and an $(m - 2)$ bp dsDNA spacer with a 2 nt ssDNA TT at the junction between the toehold and spacer domains.

The strand-displacement rate can be changed by over 3 orders of magnitude through control of the hybridization state of the spacer domains (Figure 2A). The reaction rate is highest when

dx.doi.org/10.1021/ja1073239 |J. Am. Chem. Soc. XXXX, XXX, 000–000

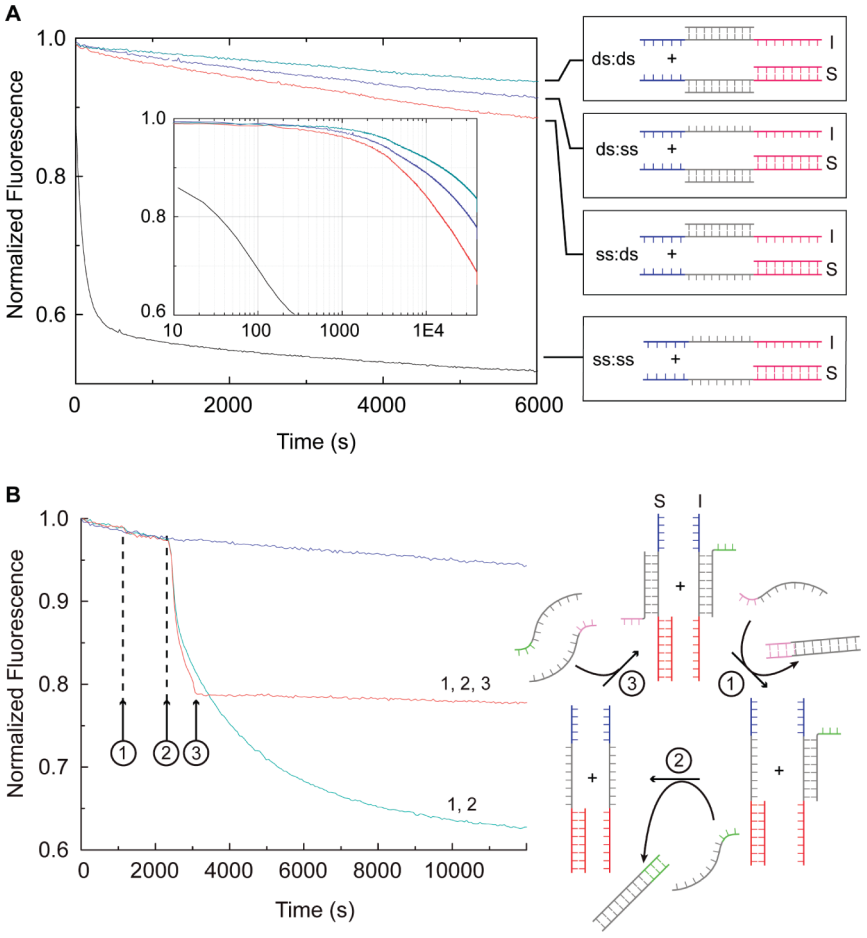
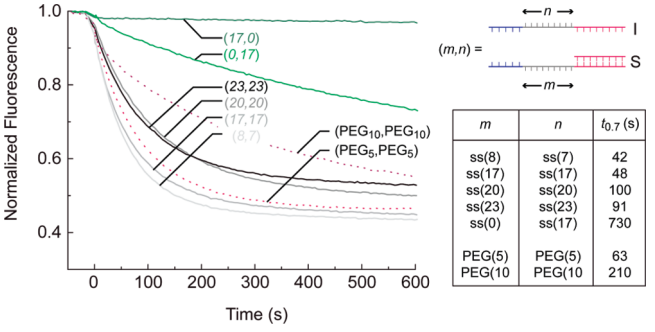


Figure 2. Control of strand-displacement rates through control of spacer hybridization. (A) Strand-displacement reactions for all combinations of 23 nt ssDNA and dsDNA spacers. Initial concentrations: [target complex] = 6.6 nM, [invading strand] = 26 nM except in the ss−ss case where [invading strand] = 133 nM. (B) Dynamic control of strand-displacement kinetics. All traces start from ds(23)−ds(23). The ds spacers may be made ss by removal of the bracing strand. The system in the top trace remains in the ds−ds state. For other traces the system is cycled as follows: ds−ds → ss−ds → ss−ss → ds−ds (middle), ds−ds → ss−ds → ss−ss (bottom). Initial concentrations: [ds−ds target complex] = 6.6 nM, [invading strand] = 13.3 nM, [substrate brace remover] = 33 nM, [invading strand brace remover] = 66 nM, [replacement bracing strands] = 133 nM.



m	*n*	$t_{0.7}$ (s)
ss(8)	ss(7)	42
ss(17)	ss(17)	48
ss(20)	ss(20)	100
ss(23)	ss(23)	91
ss(0)	ss(17)	730
PEG(5)	PEG(5)	63
PEG(10	PEG(10	210

Figure 3. Effect of the lengths of flexible spacers. Spacers are either ssDNA or 5 or 10 units of polyethylene glycol. PEG(10) consists of two PEG 5-mers separated by a phosphodiester linkage. Initial concentrations: [target complex] = 6.6 nM, [invading strand] = 660 nM. Table: time required to reach a 70% signal.

both spacers are single-stranded and therefore flexible.[28] The reaction rate is reduced by 2 or 3 orders of magnitude when one or both spacers are converted to a rigid double helix. Changing the hybridization state of the spacer domains allows dynamic, allosteric control of the strand-displacement kinetics (Figure 2B). Single-stranded spacers can be made double-stranded by adding a complementary bracing strand, and bracing strands that carry their own (proximal) toeholds can be removed by adding complementary removal strands. The remote-toehold-mediated strand-displacement rate is dramatically increased when both bracing strands are removed, and the reaction can be effectively stopped by replacing them.

Fine adjustment of the strand-displacement rate can be made by changing the spacer lengths: lengthening ssDNA spacers from 17 to 23 nt slows the displacement by about 50% (Figure 3); in the ds−ss system, ds(20)−ss(20) is slower than ds(17)−ss(17) by about 35% (Figure S4A, Supporting Information). For comparison, deletion of a single nucleotide from a proximal toehold reduces the strand-displacement rate by an order of magnitude.[5] The strand-displacement rate decreases monotonically as the spacer length increases in ds−ss and ds−ds systems (Figure S4), and the ss−ss system behaves similarly (Figure 3). However, ss(23)−ss(23) reacts more rapidly than ss(20)−ss(20); this may be caused by unintended, weak base-pairing interactions between the two spacers which cannot occur in ds−ss and ds−ds configurations. A simple model for the ds−ss system that treats ssDNA as a wormlike chain[29] accounts qualitatively for the

dx.doi.org/10.1021/ja1073239 |J. Am. Chem. Soc. XXXX, XXX, 000–000

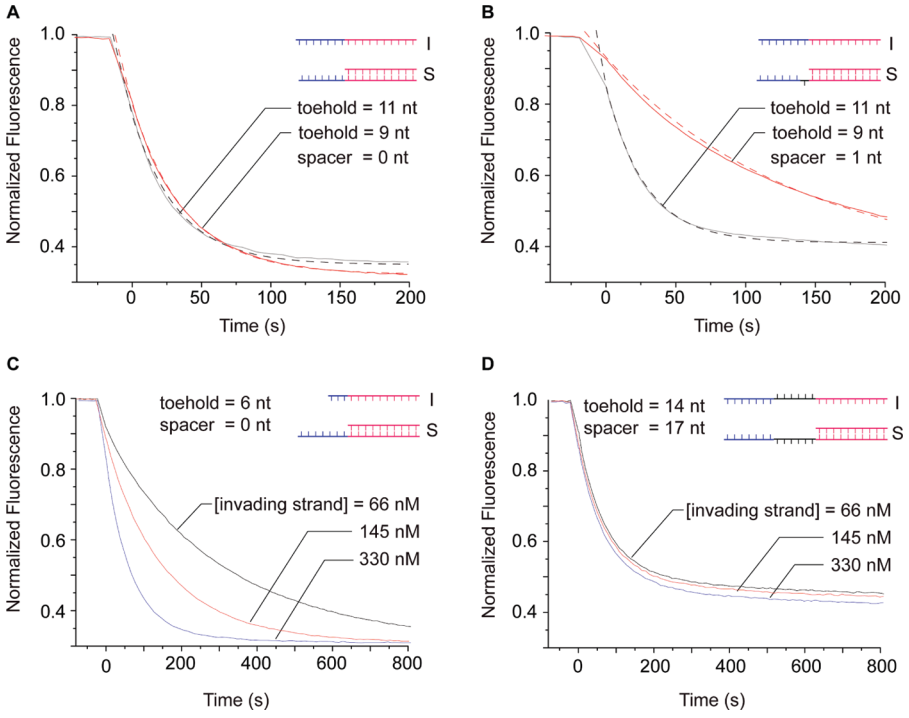


Figure 4. Kinetic proofreading (A, B) and concentration-robust (C, D) regimes. (A) The rates of toehold-mediated strand displacement using proximal 9 and 11 nt toeholds are similar (left panel). (B) Introduction of a 1 nt spacer between the toehold and displacement domains of the substrate allows effective kinetic discrimination between the same 9 and 11 nt toeholds (right panel). Initial concentrations: [substrate] = 6.6 nM, [invading strand] = 22 nM. Fitted curves correspond to a single-exponential decay. (C) With a 6 nt proximal toehold, the reaction rate depends strongly on the concentration of the invading strand (left panel). (D) By increasing the toehold length to 14 nt and inserting 17 nt spacers between the toehold and displacement domains, the reaction rate can be made insensitive to the concentration of the invading strand. Initial concentrations: [substrate] = 6.6 nM, [invading strand] = 66, 145, or 330 nM.

length dependence of the reaction rate and provides an order-of-magnitude estimate of the absolute rate (Supporting Information section 3). A comprehensive model of the kinetics of remote toeholds must incorporate a quantitative description of all interactions, including counterion-dependent electrostatic interactions, between the spacer domains.

The spacer domain need not be composed of DNA. Two of the reaction curves in Figure 3 correspond to a system in which the DNA spacers were replaced by flexible, uncharged, polyethylene glycol linkers.

The invading strand and the substrate complexes can, of course, incorporate spacers of different lengths. It is interesting to note that the ss(17)−ss(0) and ss(0)−ss(17) systems are both significantly slower than ss(17)−ss(17), despite having a shorter composite linker connecting the toehold and displacement domains. There is also a remarkable asymmetry between ss(17)−ss(0) and ss(0)−ss(17), which may result from stabilization of branch migration intermediates by stacking of the toehold and displacement domains.

The rate constant for strand displacement in the absence of any toehold, proximal or remote, has been consistently measured to be about 1 M^{-1} s^{-1}.[5,6,25] However, recent investigations of metastable DNA fuel complexes has shown that unintended (leak) strand-displacement reactions typically occur faster than this implies.[12,17,30−32] Remote-toehold strand displacement due to unintended sequence complementarity between single-stranded domains is likely to be a culprit.

3.2. Kinetic Regimes: Interplay among Concentration, Toehold Length, and Internal Displacement. The experiments presented in Figures 2 and 3 show that the rate of the internal displacement step can be controlled through control of the length and hybridization state of the spacers. The toehold docking and dissociation rates can be controlled through the concentrations of the reactants and toehold binding strength.[5,6] By manipulating the interplay among the concentration, toehold binding strength, and internal displacement rate, it is possible to design strand-displacement reactions to operate in different kinetic regimes (Supporting Information).

3.2.1. Kinetic Proofreading Regime. The rate of proximal-toehold-mediated strand displacement varies roughly exponentially with the toehold binding free energy from 0 to around −10 kcal mol^{-1} but saturates for higher binding strengths.[5,6] Proximal toehold reactions can therefore barely discriminate between toehold binding interactions involving more than 8−10 nt (Figure 4A). Introduction of a spacer between the toehold and displacement domains introduces an internal diffusion step between toehold binding and strand displacement, slowing the reaction. This extends the range of toehold binding strengths for which there is a significant probability that an invading strand will dissociate unproductively. It thus increases the energy threshold at which the reaction rate saturates with the toehold binding strength.[6] The introduction of a 1 nt single-stranded spacer in the substrate increases the kinetic discrimination between a 9 and 11 nt toehold by 3−4-fold (Figure 4B). Marked kinetic discrimination between 8 and 10 nt toeholds is also observed in the ds−ds system (Figure S5, Supporting Information).

The insertion of a spacer domain preserves the modularity of toehold-mediated strand displacement, i.e., the independence of

dx.doi.org/10.1021/ja1073239 |J. Am. Chem. Soc. XXXX, XXX, 000–000

the toehold and displacement domain sequences. It permits the reaction rate to be tuned without changing either of these domains. The use of spacers to enhance discrimination between long toeholds could greatly expand the sequence space available for the design of DNA circuits that rely on discrimination between strand-displacement rates.[8,9,21] This strategy could also enhance the discrimination of hybridization probes used to detect sequence variations, e.g., single-nucleotide polymorphisms, in natural nucleic acids.

3.2.2. Concentration-Robust Regime. The effective order of a toehold-mediated strand-displacement reaction depends both on the concentrations of the reactants and on the balance between the rates of the substeps: toehold docking and dissociation and the nucleation and completion of strand displacement.[6] Strand-displacement reactions can be made pseudo first order by ensuring that one reactant is present in sufficiently large excess. A toehold-mediated strand-displacement reaction is pseudo first order if toehold binding is effectively irreversible and sufficiently rapid that it is not rate limiting. By introducing a slow internal diffusion step, it is possible to build systems that operate in the first-order regime with a rate constant that is robust to variations in the reactant concentrations over a wide range. Strand-displacement reactions that are robust to variations in the concentrations of the reactants may improve the robustness of strand-displacement reaction networks.[8,9,13]

To demonstrate this design principle, we designed two strand-displacement systems that operate on similar time scales but with different robustnesses to the concentration of the reactants.

The concentration-robust system incorporates a remote toehold and was designed to ensure that internal displacement was slower than hybridization. The toehold is long (14 nt) to ensure that toehold binding is rapid and effectively irreversible, and the internal displacement step has been slowed by the introduction of 17 nt ssDNA spacers. Under the experimental conditions chosen, we expect the half-time for toehold hybridization[4−6] to be around 10 s, which is at least 5 times quicker than the half-time for internal displacement in an ss(17)−ss(17) system (see Figure 3). This remote-toehold system is robust to concentration variation, as designed (Figure 4D): the observed variation in the reaction rate with the concentration of the invading strand is within the experimental uncertainty.

The corresponding concentration-dependent system was designed with a proximal toehold such that the rate of second-order toehold association contributes significantly to the overall reaction rate. A proximal toehold of 6 nt was chosen to give a reaction time scale that is comparable to that of the concentration-robust system while depending strongly on the concentrations of the reactants (Figure 4C).

4. CONCLUSIONS

We have developed a mechanism that allows increased control of strand-displacement kinetics in DNA devices. The remote toehold is a generalization of toehold-mediated strand displacement and introduces additional design flexibility, including the possibility of tuning strand-displacement rates over at least 3 orders of magnitude. The remote-toehold mechanism can also cause unintended strand-displacement reactions. DNA strand displacement has been shown to be a universal primitive of arbitrary reaction networks,[13] and high-level tools are being developed to formalize and simulate strand-displacement networks.[26,33,34] Strand displacement is also the basis of many synthetic molecular machines.[14,15,17−19,35−37] We expect the additional design flexibility provided by the introduction of remote toeholds to contribute to the development of DNA-based molecular computation[9,38,40,41] and of molecular robotics.[39,42]

■ ASSOCIATED CONTENT

Supporting Information. DNA sequences, further investigation of ds−ss and ds−ds systems, a WLC chain model of ds−ss kinetics, and kinetic modeling of the remote-toehold mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

D.Y.Z. was supported by the Fannie and John Hertz Foundation and is currently a Howard Hughes Medical Institute Fellow of the Life Sciences Research Foundation. A.J.G. was supported by the Ecole Polytechnique in Paris, Merton College, Oxford, and the EPSRC (Grant EP/P501709/1 and PhD Plus Award EP/P503876/1).

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