Aptamers

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Toehold-Mediated Displacement of an Adenosine-Binding Aptamer from a DNA Duplex by its Ligand

*Jon H. Monserud[+], Katherine M. Macri[+], and Daniel K. Schwartz**

Abstract: *DNA is increasingly used to engineer dynamic nanoscale circuits, structures, and motors, many of which rely on DNA strand-displacement reactions. The use of functional DNA sequences (e.g., aptamers, which bind to a wide range of ligands) in these reactions would potentially confer responsiveness on such devices, and integrate DNA computation with highly varied molecular stimuli. By using high-throughput single-molecule FRET methods, we compared the kinetics of a putative aptamer–ligand and aptamer–complement strand-displacement reaction. We found that the ligands actively disrupted the DNA duplex in the presence of a DNA toehold in a similar manner to complementary DNA, with kinetic details specific to the aptamer structure, thus suggesting that the DNA strand-displacement concept can be extended to functional DNA–ligand systems.*

In addition to the familiar Watson–Crick base-pairing interactions, nucleic acids can fold into complex shapes that act as frameworks for specific binding to ligands other than nucleic acids.[1] This concept has been extended to include non-biological oligonucleotides known as aptamers,[2,3] which are single-stranded DNA (ssDNA) constructs that bind to predetermined targets with high affinity and specificity.[4] Compared to other molecular recognition elements such as monoclonal antibodies, aptamers can be developed in a rapid and robust manner through the systematic evolution of ligands by exponential enrichment (SELEX).[3,5] In this way, aptamers can be designed to target small organic molecules,[6] proteins,[7] and antibodies.[8] Moreover, aptamers can be prepared using solid-phase synthesis, and can interface directly with DNA technologies that involve hybridization, such as sequencing, gene chips, and DNA computation.

One important class of DNA reactions involves a process where a strand of DNA displaces another in binding to a third strand with partial complementarity to both.[9] This strand-displacement scheme can be viewed as a reaction in which an "input" strand of ssDNA reacts with a dsDNA complex to create an "output" strand of ssDNA and a different dsDNA complex. The kinetic and equilibrium properties of strand-displacement reactions are readily tuned by controlling the DNA sequences of the "reactant" molecules, which has led to the demonstration of intriguing synthetic molecular nanodevices, including DNA computers,[10–12] molecular motors,[11,13] and catalytic cycles.[11,14] Strand-displacement reactions require a molecular feature commonly known as a "toehold", a short single-stranded overhang. An input strand is believed to bind to the exposed toehold and then increase the length of the hybridized region through a mechanism known as branch migration, ultimately completing the displacement process. Importantly, the macroscopic rate of a strand-displacement reaction depends on the toehold length, thus allowing kinetic control of these reactions; this is a signature of systems that depend on toehold exchange processes.[15]

In principle, aptamers could provide a way to interface DNA strand displacement reactions with the wider world of chemical signals.[12] Specifically, if one could incorporate aptamer displacement by a chemical ligand into a DNA strand-displacement reaction network, new applications would be enabled. Previous work has explored the possibility of ligand-mediated aptamer displacement using quencher-based fluorescence spectroscopy,[16] and it was found that in the presence of a ligand, the concentration of the aptamer–DNA duplex was reduced. Because these were ensemble-averaging measurements, it remains unclear whether these observations were simply the result of a shift in the binding equilibrium caused by the introduction of a competitive binder for the aptamer, or whether the process involved active dynamic toehold exchange. The latter would permit rational control of the reaction rate, a critical feature of many proposed technologies.[9,15,17] The experiments described herein directly address these issues at the single-molecule level, providing direct information about the kinetics of the elementary processes.

We used single-molecule Förster resonance energy transfer (FRET) to measure the dynamics of adenosine aptamer[6] hybridization and dehybridization with a complementary DNA "capture" strand that was immobilized at the interface between aqueous solution and modified fused silica.[18] We hypothesized that the rates of dehybridization in the presence or absence of the adenosine ligand would directly probe the nature of the aptamer–ligand and aptamer–DNA competition. Similar experiments in the presence or absence of DNA complementary to the aptamer have provided analogous information about the kinetics of the corresponding DNA strand-displacement reactions. To assess the role of the toehold, four different acceptor-labeled immobilized capture strands were used: one that was fully complementary to the aptamer (affording no toehold) and three successively shorter

[*] J. H. Monserud,[+] K. M. Macri,[+] D. K. Schwartz
Department of Chemical and Biological Engineering
University of Colorado Boulder, Boulder, Colorado 80309 (USA)
E-mail: daniel.schwartz@colorado.edu
[+] These authors contributed equally to this work.

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capture strands that were partially complementary to the aptamer sequence, exposing a 0-, 6-, 11-, and 16-base toehold when hybridized to the adenosine aptamer (0bT, 6bT, 11bT, 16bT, respectively). These were exposed to an aqueous phase containing a low concentration (100 pM) of the adenosine aptamer modified with a FRET donor in an appropriate location to achieve FRET upon hybridization, as illustrated in Figure 1. The labeled aptamer molecules adsorbed randomly

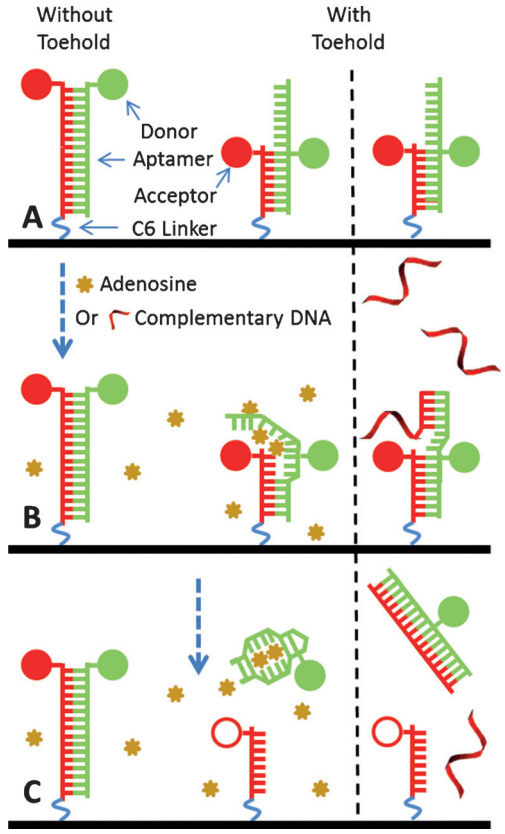

Figure 1. Schematic diagram of the experimental systems, illustrating the physical interpretation of the observed phenomena. Donor fluorophores are shown in green and acceptor fluorophores in red.

and diffused over the surface, potentially locating and binding to a complementary strand. The duplex DNA was formed with or without a toehold (Figure 1 A) depending on the length of the capture strand. These interactions were highly dynamic and reversible, thus permitting us to measure the distributions of time intervals associated with the hybridized state based on a distinctive FRET signature.[19]

To probe the influence of toehold length on exchange kinetics, dynamic single-molecule tracking experiments were performed in the presence and absence of (2 μM) adenosine (Figure 1). In these experiments, reduction of the time intervals for the hybridized state in the presence of adenosine, compared to experiments in the absence of adenosine, indicate active strand displacement by the ligand. For comparison with more traditional strand-displacement processes, these experiments were repeated using DNA complementary to the adenosine aptamer (100 pM) as the "ligand." Dual-channel image sequences were acquired,[18,19] comprising more than 600 000 molecular trajectories for each capture strand. Additional control experiments were performed using donor-labeled non-complementary poly-A oligonucleotides as "ligands". Surface modification, oligonucleotide sequences and modification, and FRET-TIRFM experiments are described in the Supporting Information (TIRFM = total internal reflection fluorescence microscopy).

The fraction of freely-adsorbing aptamer molecules that successfully hybridized to the immobilized capture strands (f_H) was measured in the presence and absence of ligand. The presence of adenosine reduced f_H by 50% for all aptamers, which is consistent with the existence of a pre-formed aptamer–ligand complex. Since adenosine was added at a concentration corresponding to the dissociation constant of the unlabeled aptamer, it was expected that half of the aptamer molecules would exist in bound form. Notably, this suggests that the affinity of the aptamer was unaffected by labeling. Quantitative details are given in Table S1 in the Supporting Information. Each hybridization event was dynamic, and ultimately ended through transition back to surface diffusion or to desorption as described previously.[19] Figure 2 shows complementary cumulative probability distributions for the time intervals associated with the hybridized state,[20] which indicate the fraction of aptamer molecules remaining hybridized to capture strands for time t or longer. These distributions were non-exponential, thus suggesting the presence of multiple populations with distinct characteristic state times, and were fitted to an exponential mixture model.[21] The characteristic hybridization times and their respective population fractions were extracted from these fits and used to calculate a weighted average to determine the mean association times. By comparing these results to those using a poly-A negative control that was incapable of hybridization, a ubiquitous but short-lived (<1 s) population was identified that corresponded to incidental collisions, thus allowing the separation of longer-lived true hybridization events.[21] As expected, we observed that the mean association lifetimes of duplex DNA (in the absence of adenosine) decreased systematically with increasing toehold length, from 1.9 s to 1.2 s, since shorter capture strands had less complementarity with the aptamer. Similarly, the mean lifetimes of the longer-lived modes decreased from 6.5 s to 5.2 s. A more detailed discussion of the calculation and analysis of these distributions is provided in the supporting information, including fit parameters in Table S2.

For the complementary capture strands that exhibited an exposed toehold, the hybridized state lifetimes were generally reduced (with one important exception as discussed below) in the presence of adenosine or the aptamer-complementary DNA compared to experiments in the absence of ligands (Figures 2 B–D). In contrast, for the fully complementary capture strand (0bT), the presence of ligand did not change the lifetime distribution of the hybridized state (Figure 2 A). This provides explicit evidence for the direct active displacement of the aptamer through ligand binding, thus suggesting that ligands can precipitate strand-displacement reactions of aptamers in analogy with typical ssDNA-mediated strand

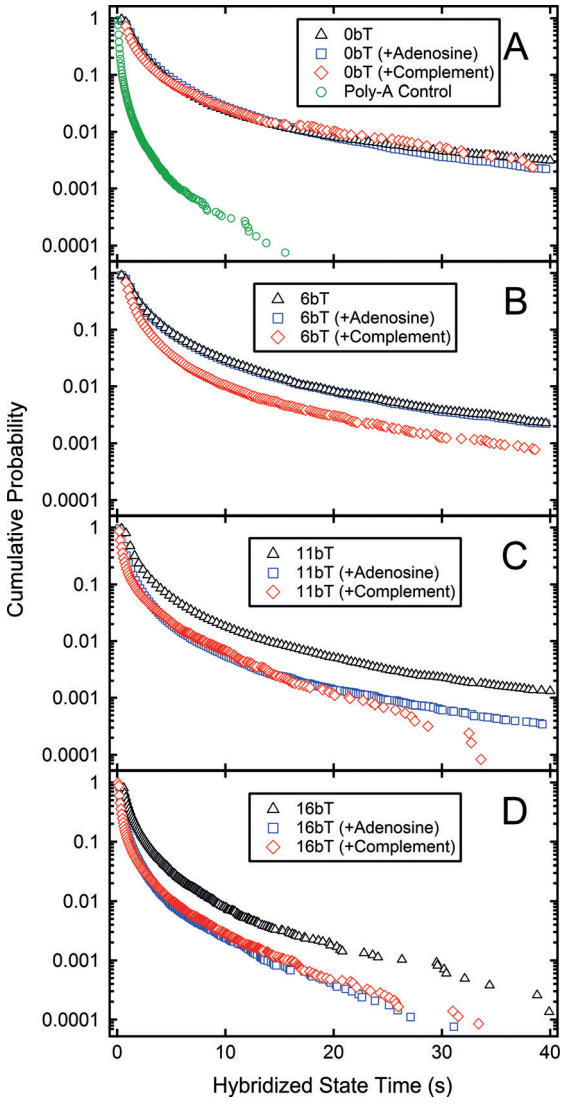

Figure 2. Cumulative hybridized-state time distributions in the absence of a ligand (△), in the presence of adenosine (■), or in the presence of DNA complementary to the aptamer (◇) for the capture stands designed to expose toeholds of 0 (a), 6 (b), 11 (c), or 16 (d) bases. The poly-A control (×) was used in the place of the adenosine aptamer as a negative control. Error bars were omitted to enhance the clarity, but are included in Figure S1 in the Supporting Information.

displacement reactions, presumably by reducing the energy barrier for dehybridization.

To demonstrate the generality of this phenomenon, analogous experiments were performed with another aptamer–ligand system, using an aptamer that binds to the organophosphate pesticide phorate (see Figure S2 and Table S4 in the Supporting Information for details). As for the adenosine-binding aptamer, hybridized-state lifetimes for the phorate-binding aptamer were reduced significantly when the relevant ligand was added in the presence of a toehold. However, for the fully complementary capture strand, the presence of the ligand had a negligible impact on the distribution of hybridization lifetimes.

A careful inspection of the lifetime distributions revealed quantitatively different trends with toehold length for experiments performed in the presence of complementary DNA versus those involving the adenosine ligand. In particular, for the 6-base toehold, association times were reduced much more by the presence of complementary DNA than by adenosine. However, for longer toeholds, the reduction in the lifetimes was similar for the complementary DNA and the adenosine "ligands". To illustrate this explicitly, the "relative mean association time" was calculated as the ratio of the mean association time in the presence of each ligand (τ_{ligand}) to that in the absence of a ligand (τ_0), and plotted as a function of toehold length (Figure 3). As suggested by the lifetime

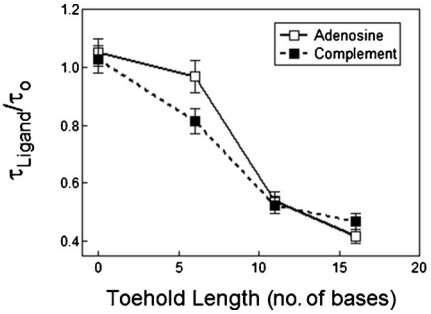

Figure 3. Hybridization times in the presence of ligand normalized by hybridization times in the absence of ligand for each toehold length. Error bars were calculated as the standard deviation of the mean hybridization times obtained from movies captured at six independent locations on each of triplicate samples for each experimental condition (as described in the Supporting Information).

distributions, in the presence of the complementary DNA, a continuous (nearly linear) decrease in relative association times was observed with increasing toehold length, a trend that is qualitatively consistent with previous reports of ensemble-averaged kinetics.[22] In contrast, no significant response to the adenosine ligand was observed for the 0bT and 6bT systems, followed by a dramatic reduction in hybridization times to mimic the behavior of the complementary DNA ligand for longer toeholds.

Similarly, a detailed analysis employing the parameters associated with the lifetime distribution fits indicated that the characteristic timescales of individual hybridization modes were directly influenced by the presence/absence of adenosine. In particular, the timescale related to longer associations (i.e. true hybridization) was determined by calculating a weighted average of the longer-lived modes. For the 0bT capture strand, this mean hybridized state lifetime was insensitive to the presence of ligand. For 11bT and 16bT toeholds, reduction in true hybridization times of more than or equal to 45 % occurred when either ligand was introduced. However, for the 6bT system, there was a reduction in hybridization times in response to the complementary DNA, but not to the adenosine ligand.

We hypothesized that this discontinuous dependence on toehold length was related to the degree of exposure of the adenosine aptamer "active site" when hybridized to capture DNA of different lengths. The proposed adenosine–aptamer complex involves a framework comprising two stacked G-quadruplexes[6] (Figure 4C). The 0bT and 6bT capture-strand

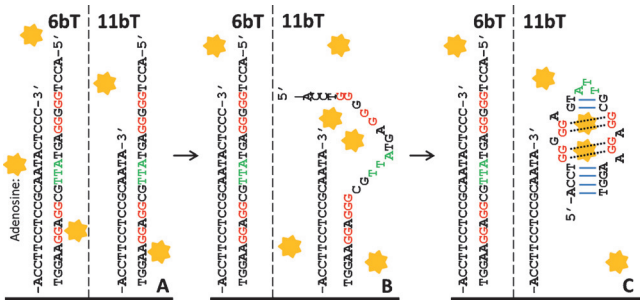

Figure 4. A schematic representation of strand displacement by the ligand for the adenosine aptamer, illustrating the physical interpretation of the observed phenomena. G-quadruplex bases are highlighted in red. The three-base loop is highlighted in green.

duplexes block both halves of the G-quadruplex structure, which begins five bases from the 5′ end (Figure 4A). In contrast, the 11bT and 16bT capture-strand duplexes expose half of the bases that form the G-quadruplex, thereby allowing adenosine molecules to interact with the exposed active site and actively displace complementary DNA (Figure 4B). Moreover, when hybridized to the 6bT capture strand, the bases forming the stem-loop secondary structure of the active aptamer are conformationally constrained (Figure 4A), thus greatly limiting the ability of the aptamer ability to conform to the adenosine ligand. These combined observations suggest that aptamer–ligand strand-displacement reactions require exposure of the aptamer "active site" in the toehold as well as sufficient flexibility to conform to the ligand. This results in a step-wise dependence of ligand-induced strand displacement on toehold length.

Single-molecule tracking has provided a unique opportunity to probe the dynamic behavior of aptamer–ligand toehold exchange in an environment relevant to DNA computing, sensing, nanomachines, and other DNA nanotechnologies. While aptamers fully-hybridized to surface-immobilized DNA melted prior to forming aptamer–ligand complexes, we found that the ligand actively disrupted partially hybridized aptamers by interacting with toeholds that exposed the active site of the aptamer. This process exhibits remarkable similarity to the DNA-DNA toehold exchange systems used in a variety of DNA based devices, thus suggesting that non-DNA chemical signals can be interfaced with active DNA technology in a dynamically controllable manner.

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