Single-Molecule Fluorescence Imaging of Interfacial DNA Hybridization Kinetics at Selective Capture Surfaces

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Supporting Information

ABSTRACT: Accurate knowledge of the kinetics of complementary oligonucleotide hybridization is integral to the design and understanding of DNA-based biosensors. In this work, single-molecule fluorescence imaging is applied to measuring rates of hybridization between fluorescently labeled target ssDNA and unlabeled probe ssDNA immobilized on glass surfaces. In the absence of probe site labeling, the capture surface must be highly selective to avoid the influence of nonspecific adsorption on the interpretation of single-molecule imaging results. This is accomplished by increasing the probe molecule site densities by a factor of ~100 compared to optically resolvable sites so that nonspecific interactions compete with a much greater number of capture sites and by immobilizing sulfonate groups to passivate the surface between probe strands. The resulting substrates exhibit very low nonspecific adsorption, and the selectivity for binding a complementary target sequence exceeds that of a scrambled sequence by nearly 3 orders of magnitude. The population of immobilized DNA probe sites is quantified by counting individual DNA duplexes at low target concentrations, and those results are used to calibrate fluorescence intensities on the same sample at much higher target concentrations to measure a full binding isotherm. Dissociation rates are determined from interfacial residence times of individual DNA duplexes. Equilibrium and rate constants of hybridization, $K_0 = 38 \pm 1 \mu M^{-1}$, $k_{on} = 1.64 \pm 0.06 \times 10^8 M^{-1} s^{-1}$, and $k_{off} = 4.3 \pm 0.1 \times 10^{-2} s^{-1}$, were found not to change with surface density of immobilized probe DNA, indicating that hybridization events at neighboring probe sites are independent. To test the influence of probe-strand immobilization on hybridization, the kinetics of the probe target reaction at the surface were compared with the same reaction in free solution, and the equilibrium constants and dissociation and association rates were found to be nearly equivalent. The selectivity of these capture surfaces should facilitate sensitive investigations of DNA hybridization at the limit of counting molecules. Because the immobilized probe DNA on these surfaces is unlabeled, photobleaching of a probe label is not an issue, allowing capture substrates to be used for long periods of time or even reused in multiple experiments.

Base pair interactions between short oligonucleotides play a critical role in the emerging fields of nucleic acid-based chemical assays, drug therapy, and nanotechnology. Sequence-specific base pair interactions govern the behavior of DNA and RNA aptamers used in analytical assays, oligonucleotide-based therapeutics, and drug delivery systems, fluorescence in situ hybridization cell imaging, and emerging self-assembly in nanotechnology, including 2D and 3D DNA origami structures and directed nanoparticle assemblies. For controlling DNA nanostructure assemblies and the behavior of small nucleotides, aptamers, and therapeutic agents, the fundamental role that experimental variables play in these assays must be understood in terms of the energetics and kinetics of oligonucleotide hybridization.

Probe molecule immobilization is widely used for measuring biomolecular interactions; it provides in situ separation of target analytes from the bulk sample and is compatible with a variety of surface-sensitive detection schemes. DNA hybridization has been detected using total internal reflection fluorescence (TIRF), surface plasmon resonance, microring resonators, and plasmonic nanostructure-enhanced fluorescence assays. Although these techniques are capable of elucidating association equilibrium constants by determining relative target surface coverages, measuring hybridization kinetics is more challenging. First, the measured spectroscopic response is difficult to convert to absolute molecular surface concentrations, which are needed to report hybridization rates. Additionally, association kinetics in these assays are typically measured using concentration-step experiments, where slow mass transport of molecules from low-concentration solutions to surfaces with high densities of capture sites dominates the transient response.

Improvements in the detection efficiency of TIRF microscopy have allowed imaging of individual fluorescently labeled molecules binding to probes immobilized on a surface at...
equilibrium in real time. By making measurements at equilibrium, the on- and off-rates are no longer limited by mass transport during a concentration jump or wash off step. Researchers have observed hybridization of individually bound DNA duplexes at very low surface densities, where the individual DNA capture sites can be resolved spatially using fluorescence microscopy.\(^{20-27}\) A challenge in these measurements is that binding sites must be spaced >500 nm apart to be resolved optically, resulting in a very small (~10\(^{-6}\)) fraction of the surface area being occupied by DNA probe sites. Because the vast majority of the surface contains no probe DNA, nonspecific target DNA adsorption to the interstitial area can dominate the population of molecules on the surface.\(^{25}\) Additional means are needed to differentiate hybridized versus nonspecifically adsorbed target DNA, which have included colocalization of the target with probe DNA strands on fluorescently labeled DNA origami\(^{21}\) or detection of fluorescence (Förster) resonance energy transfer (FRET) between donor and acceptor fluorophores on probe and target strands.\(^{23,25}\) These labeled probe schemes add complexity to single-molecule experiments and present some disadvantages. Co-localization on labeled origami is complicated by microscope stage drift between the localization of probe strands and the identification of hybridized target molecules. Additionally, hybridization on DNA origami has been shown to be heterogeneous, where hybridization rates depend on the location of ssDNA on the origami structure.\(^{25,27}\) FRET experiments are hindered by photobleaching of the surface-bound fluorescent label on the probe strand, limiting the number of hybridization events that can be observed at any probe site. In addition, FRET donor and acceptor fluorophores placed in close proximity in dsDNA have been shown to influence the energetics of hybridization.\(^{28}\)

The goal of this work is to develop a single-molecule approach to measuring equilibria and kinetics of DNA hybridization by detecting fluorescence only from a labeled target ssDNA. By using unlabeled immobilized probes, costly steps of probe labeling are avoided, producing substrates that can be used for long periods of time or even reused for multiple experiments, because photobleaching of a probe label is not an issue. Without probe site labeling to identify authentic hybridization events, however, nonspecific adsorption must be negligible compared to probe site binding. Our strategy to accomplish this goal is two-fold: first, we increase the probe site densities by approximately two orders of magnitude compared to optically resolvable sites, so that nonspecific interactions must compete with a much greater number of capture sites. Second, we developed chemistry to passivate the surrounding surface to nonspecific DNA adsorption to increase the selectivity for detecting the hybridized population and to reduce surface interactions that could influence the dynamics and stability of base-pairing interactions.\(^{25,29,30}\) The selectivity of the resulting surfaces is tested by measuring their affinities for a complementary target strand and comparing the results to a scrambled sequence of comparable base composition. Target-probe interactions are sequence-specific and quantified by counting individual hybridized target DNA molecules at the surface; these results are used to calibrate a fluorescence intensity isotherm at higher coverages to determine the surface density of probe DNA sites. Dissociation kinetics are measured by tracking individual target probe hybridization events. Hybridization rates are measured versus probe site density to test whether capture and dissociation at neighboring probe sites are independent. The influence of the passivated surface on the stability of the dsDNA duplex is tested by direct comparison with the hybridization equilibria and kinetics of the same probe target reaction in free solution.

## EXPERIMENTAL SECTION

**Labeled Oligonucleotides.** Chemically modified oligonucleotide probes for immobilization and targets and controls for fluorescence detection were synthesized using solid-phase phosphoramidite chemistry and cartridge purified by the University of Utah HSC Core DNA synthesis facility. Target ssDNA was HPLC purified to remove free fluorescent dye. The immobilized 5’s-amine-modified probe sequence has a 15-base sequence: NH\(_2\)-C\(_6\)H\(_{12}\)-5’-GTCGGTATATCCCAT-3’. Fluorescently labeled targets have sequences complementary to the final 10 or 12 bases on the immobilized probe: Cy3-PEG\(_2\)-5’-ATGGGATATA-3’, and Cy3-PEG\(_3\)-5’-ATGGGATATACC-3’, respectively. A control “scrambled” 10-mer ssDNA has similar base composition but little overlap with the probe: Cy3-PEG\(_2\)-5’-AGTAGTAGAT-3’. Chemical sources and structures of DNA target, control, and probe strands are listed in the Supporting Information.

**Probe-ssDNA Immobilization.** To prepare substrates for DNA immobilization, glass coverslips were first cleaned in piranha solution, 1:3 30% H\(_2\)O\(_2\):96% H\(_2\)SO\(_4\). A control (caution: corrosive, strong oxidizer, can react explosively with organics) for 20 min. The coverslips were then thoroughly rinsed with water and cleaned in a 70 °C RCA base bath, \(^{31}\) 1:1:5 30% H\(_2\)O\(_2\):5% NH\(_4\)OH:water, for 20 min. Slides were then rinsed with deionized water, dried, and placed upright in a sealed jar with 250 μL of 3-glycidoxypropyltrimethoxysilane (GOPTS).\(^{32}\) The jar was heated to 60 °C in an oven with fine temperature control (±0.1 °C), which allowed vapor-phase deposition of the silane over a period of 16 h.

Amine-modified probe ssDNA was covalently linked to the glycidoxy silane-modified substrates using methods adapted from several sources.\(^{34-36}\) For maintaining high probe DNA concentrations during the immobilization reaction, a small-volume scheme was developed. A 10 μL drop of 125 to 375 μM amine-ssDNA in 100 mM pH 10 carbonate buffer was sandwiched between a silane-modified and a clean glass coverslip. The coverslips were heated in humidity-controlled closed jars at 40 °C for 12 to 24 h. Amine-ssDNA concentrations and reaction times were varied to achieve different probe DNA surface densities. The coverslips were removed, rinsed with water, and placed in pH 10 carbonate buffer containing 40 mM surface-passivation reagent, 3-amino-1-propanesulfonic acid at 40 °C for 20 h. Blank passivated surfaces were generated by exposing GOPTS-modified surfaces to pH 10 carbonate buffer (no amine DNA) for 2 h at 40 °C followed by exposure to pH 10 carbonate buffer with 40 mM 3-amino-1-propanesulfonic acid at 40 °C for 20 h. Reaction schemes for DNA immobilization and surface passivation are in the Supporting Information.

**TIRF Microscopy.** DNA-modified coverslips were assembled into an imaging flow cell consisting of a 145 μm thick acrylic polyester double-stick gasket (3M, 9495MPF) sandwiched between the coverslip and a glass top-plate with sample inlet and outlet ports. A 10 mm-by-3 mm channel cut into the gasket connected the top-plate inlet and outlet ports. Buffer solutions with 2 mM pH 7.4 phosphate, 250 mM sodium chloride, and variable concentrations of target ssDNA were flowed through the channel at a rate of 0.1–0.3 mL min\(^{-1}\) with

DOI: 10.1021/acs.analchem.5b03832

a syringe pump. Flow rate had no significantly impact on measured association kinetics with measured $k_{on} = 1.6 \times 10^{6} \pm 0.1 \times 10^{6}$ and $1.67 \times 10^{6} \pm 0.08 \times 10^{6} \text{M}^{-1} \text{s}^{-1}$ at 0.1 and 0.2 mL min$^{-1}$, respectively, indicating that the association rate is not affected by solution flow in this range. Experiments were conducted at 22.6 ± 0.2 °C. Hybridization was monitored in situ using an Olympus IX-71 inverted microscope equipped with a home-built through-the-objective TIRF illumination system. TIRF illumination is achieved with a 532 nm laser beam (B&W Tek BWN-532-50E) coupled into a single-mode polarization-maintaining optical fiber (Thorlabs P3-488PM-FC-2). Light exiting the fiber is collimated with a 60 mm achromatic doublet (Thorlabs) and directed through a quarter-wave plate (Thorlabs WPQ10M-532). Excitation is reflected from a dichroic mirror (Semrock FF555-Di02) and focused with a 150 mm achromatic doublet lens (Thorlabs) onto the back focal plane of the oil-immersion microscope (60× PlanApo TIRFM 1.45NA, Olympus). Translating the optical fiber normal to the optical axis shifts the angle of illumination until total internal reflection is produced at the glass-aqueous solution interface. Fluorescence emission is collected and passed through an emission band-pass filter centered at 582 nm (Semrock FF01-582/75-25) followed by a 1.6× magnifier (Olympus) to increase the magnification to 96×.

Fluorescence images are collected with an Andor iXon DU897 charge-coupled device using 200 ms integrations in 2.0 s time-lapse intervals at a 60× electron-multiplying gain and readout of 1 MHz for a 256-by-256 pixel region, corresponding to 43-by-43 μm at the sample. Excitation intensity was kept low, 2.2 mW (3 W cm$^{-2}$ during illumination, 0.3 W cm$^{-2}$ averaged over 2 s intervals) to minimize photobleaching (Supporting Information). For measuring hybridization isotherms, the average fluorescence intensity was determined by summing pixel charge within the same 43-by-43 μm region used to image single molecules. For fluorescence intensity measurements, the camera was operated with electron-multiplying gain deactivated. A second microscope capable of imaging at higher framing rates and excitation-power densities was used for quantifying target-ssDNA at passivated surfaces (see Supporting Information). All images were acquired and stored as 16-bit monochrome TIF-stacks using Andor SOLIS software version 4.24.30003.0.

Image Analysis for Counting and Tracking Single Molecules. Image analysis was performed using programs written in Matlab 2012b (Mathworks). Single-molecule spots were located in images using a threshold-based algorithm utilizing spatial information. In this method, molecular intensity spots having a size consistent with the point spread function (PSF) are identified by locating regions with three or more adjacent pixels above a threshold set at 6X the standard deviation of the background intensity. This threshold kept the false positive events below 0.01 per video frame, a small fraction of the 50–800 molecules detected in an image while minimizing false negative probabilities to <2% (see Supporting Information). The average and standard deviation of the bound-target DNA population were determined by counting molecules in images taken at intervals of twice the average dissociation time so that less than 15% of bound target molecules were sampled more than once. Observing populations at intervals of two lifetimes apart represents a compromise between measuring independent populations of molecules and making sufficient observations of the population to accurately estimate its mean and standard deviation. Uncertainties for surface populations and dissociation constants are reported as plus/minus one standard deviation of the average of at least 3 measurements.

Residence times for each detected DNA molecule were measured by tracking its centroid position in sequential video frames; brief off-events were bridged to correct for photobleaching of the fluorescent label (see Supporting Information). Low concentrations of target ssDNA (5–250 pM, depending on probe density) were used in residence-time measurements to keep bound duplexes spatially separated to prevent a neighboring event from being combined into a single residence time (<5% probability). Cumulative histograms of target DNA residence times are reported, where the count is incremented for as long as a target molecule remains bound at the surface. A cumulative residence time histogram is the integral of a discrete residence time histogram, which greatly reduces noise on the tail of the distribution. Uncertainties in reported rates were estimated by breaking the pool of molecular visits into 5–6 subpopulations and determining the dissociation rate of each subpopulation. Uncertainties in rates are reported as plus/minus one standard deviation of the average.

Hybridization Measurements in Free Solution. The fluorescence quantum yield of Cy3 attached to single-stranded DNA has been shown to be sensitive to hybridization state (single-stranded versus duplex). This behavior provides a means of monitoring probe-target hybridization in free solution by measuring Cy3-target fluorescence intensity changes in real time. A Hitachi F-7000 fluorimeter, with a Quantum Northwest TC125 stirring cuvette holder temperature controlled at 22.6 °C, was used to monitor hybridization between 10-mer fluorescently labeled target and the 15-mer probe ssDNA in free solution in the same 250 mM ionic-strength buffer used for single-molecule imaging. The complementary probe strand used in the free solution measurements has the same sequence as the tethered probe lacking only the 5′ hexadecylamine modification used for surface attachment. Fluorescence from a volume $V_t = 2 \text{ mL}$ of 2 nM target ssDNA (DNA$_t$) solution was monitored continuously with excitation and emission wavelengths of 515 and 565 nm and slit widths of 10 and 20 nm, respectively. Upon addition of probe-ssDNA at concentrations above the dissociation constant, $K_d = K_{d,1}^{-1} \approx 26 \text{ nM}$, target ssDNA fluorescence intensity increased ~10%, allowing in situ detection of hybridization (see Supporting Information). For monitoring hybridization, fluorescence intensity was monitored continuously with 0.2–0.5 s time resolution, and small aliquots (1–30 μL) of concentrated 10 μM probe were added stepwise to the rapidly stirred cuvette, resulting in final probe concentrations of [DNA$_p$] = 5–300 nM. After each stepwise addition of DNA$_p$, the transient fluorescence response was monitored for ~80 s, allowing hybridization to reach equilibrium before the next aliquot of DNA$_p$ was added. Before analysis, fluorescence time traces were coadded into 1 s bins to improve the signal-to-noise ratio. Association and dissociation rate constants were determined by fitting a relaxation-kinetic model to the transient fluorescence response, whereas association constants were determined by fitting an isotherm to the steady-state fluorescence intensities.

To account for the reduction in fluorescence intensity due to dilution of [DNA$_p$] upon each probe addition, the intensity was scaled by the factor $(V_t + V_p)/V_t$. Furthermore, [DNA$_p$] must be adjusted to account for depletion upon duplex formation. A 2 nM concentration of DNA$_i$ was needed to achieve acceptable
signal-to-noise in measured fluorescence intensity, resulting in 
\( \sim 5\% \) dilution of the [DNA<sub>p</sub>] at the highest target concentration. Depletion was included in fitting the equilibrium model (see Supporting Information) to determine \( K_a \) and then used to correct [DNA<sub>p</sub>] for loss from duplex formation, 
\[ \text{[dsDNA]} = \text{[DNA]}_t \times K_a \text{[DNA]}_p/(1 + K_a \text{[DNA]}_p) \]. This correction was applied iteratively 3 times, after which \( K_a \) and [DNA<sub>p</sub>] did not change.

**RESULTS AND DISCUSSION**

**Sequence-Specific Hybridization to Immobilized Probe ssDNA.** Substrates with immobilized probe ssDNA were equilibrated with fully complementary 10-mer target ssDNA in 250 mM ionic strength buffer and compared with a scrambled ssDNA sequence. As shown in Figure 1, the surface population of DNA observed upon equilibration with a 50 pM solution of complementary target 10-mer is 70-fold greater than the population arising from a 500 pM solution of the scrambled ssDNA, indicating selective strand recognition. The higher concentration of scrambled ssDNA was needed to achieve surface populations high enough to quantify, and the 10-fold lower concentration of complementary ssDNA was required to keep the bound molecular density sufficiently low to count molecules. Because the hybridization isotherm is linear at low concentrations (see next section), complementary target 10-mer ssDNA exhibits a 700-fold greater affinity than the scrambled sequence, indicating that nonselective interactions contribute a negligible (<0.2%) fraction to the observed surface populations. Molecule populations are similar for complementary target ssDNA at a blank sulfonate surface (no probe ssDNA), ~16 molecules per 43×43 μm field-of-view (FOV), as for scrambled ssDNA at a probe-containing surface, ~10 molecules/FOV; therefore, both results may arise from non-specific interactions with surface defect sites. With such high selectivity, more elaborate readout schemes, such as colocalization<sup>24</sup> or FRET<sup>25</sup>, are not needed to distinguish sequence-specific hybridization from non-specific interactions. By detecting only labeled target strands that are continually replenished from solution, we avoid probe photobleaching and are able to continuously observe hybridization events for long periods of time. This improves measurement statistics and allows day-long experiments interrogating the same immobilized probe population.

**Calibrating Target Isotherms to Measure Probe Density and Hybridization Equilibria.** For determining hybridization equilibrium constants from the hybridized population of target DNA, an equilibrium site binding model is used for the reaction between surface-immobilized probe ssDNA and solution-phase target ssDNA

\[
\frac{\text{[DNA]}_p}{[\text{DNA}]_t} = \frac{K_a}{1 + K_a \text{[DNA]}_p}\]

where \( \text{[DNA]}_p \) is the surface density of hybridized dsDNA, \( K_{\text{max}} \) is the surface density of probe ssDNA, \( K_a \) is the association constant, and [DNA<sub>t</sub>] is the concentration of target ssDNA in solution. At low target DNA concentrations, [DNA<sub>t</sub>] \( \ll K_a^{-1} \), eq 1 simplifies to a linear relationship

\[
\text{[DNA]}_p \approx K_{\text{max}} K_a [\text{DNA}]_t
\]

where the bound duplex population is linear with concentration of target DNA in solution, with a sensitivity that depends on the surface density of probe sites, \( K_{\text{max}} \). This relationship was tested on three separate substrates having different probe-DNA coverages, \( K_{\text{max}} \). The surface populations of duplex DNA, \( \Gamma \), were measured for [DNA<sub>t</sub>] ranging from 0.015 to 1.0 nM, and the results are plotted in Figure 2A. As expected, the responses to target concentration are linear for all samples, and the slopes of the calibration lines increase with higher density of bound probe molecules.

Although linearity of the single-molecule isotherm response is advantageous for analytical applications, these results cannot be used to determine association constants because the slope depends on the product, \( K_{\text{max}} K_a \). For resolving these two factors, the duplex coverage, \( \Gamma \), must approach \( K_{\text{max}} \) where the roll-over in \( \Gamma \) can be fit to eq 1, allowing the two parameters to be resolved. The highest coverages of bound duplex DNA in Figure 2A, \( \sim 4 \times 10^7 \) cm<sup>-2</sup>, are near the maximum that can be counted without significant (>5%) overlap of the point-spread-functions of individual molecules.<sup>37</sup> For measurements of bound duplex densities approaching \( K_{\text{max}} (>100 \times \text{the limit of resolving single-molecule spots}) \), an approach other than single-molecule counting is needed. Detecting fluorescence intensity with total internal reflection excitation is a common approach to monitoring high densities of fluorophore-labeled molecules at interfaces.<sup>31</sup> It is difficult to quantify surface populations using fluorescence intensity, however, because of uncertainties in light collection, excitation, and fluorophore photophysics. We overcome this difficulty by measuring a TIRF intensity isotherm at high target concentrations with the same sample and instrument used to count single molecules. When populations exceed the resolution limit of single-molecule counting, the TIRF microscope can seamlessly transition from single-molecule counting to measuring fluorescence intensities in the same region of the sample.

When using TIRF intensity to quantify surface-bound molecules in equilibrium with a free solution population, solution-phase target molecules in the evanescent wave can
Figure 2. Single-molecule calibrated hybridization isotherms. (A) Single-molecule hybridization isotherms for 10-mer target binding to surfaces prepared with 125 μM (cyan), 250 μM (blue), and 375 μM (black) amine probe DNA. (B, C) Hybridized target populations derived from single-molecule counting (filled squares) and fluorescence intensities (open circles) calibrated to the single-molecule response and fit to eq 1 plotted on logarithmic (B) and linear (C) scales.

The corrected fluorescence intensities (Figure 2B) thus measured were then converted to surface densities using the single-molecule population data as standards, multiplying the linear part of the intensity isotherm (100–1000 pM) by a scaling factor so that its slope matches that of the single-molecule data in Figure 2A; note that single-molecule counting and intensity data overlap in regions where both can be measured. These calibrated surface densities are fit to eq 1 to determine $\Gamma_{\text{max}}$ and $K_a$, and the results are plotted in Figure 2B and C. The probe densities, $\Gamma_{\text{max}}$, show an expected increase from $0.96 \pm 0.05 \times 10^9$ to $5.0 \pm 0.1 \times 10^9$ cm$^{-2}$, as probe reagent concentrations were increased in the immobilization reaction. The 10-mer association constants for the three surfaces, $K_a = 39 \pm 3$, 38 $\pm 2$, and 39 $\pm 1$ μM$^{-1}$, are within their uncertainties and thus independent of the density of probe sites.

An alternate means of mitigating background fluorescence from solution is to increase the strength of hybridization (increase $K_a$) so that lower solution-phase concentrations can be used to saturate the probe sites. It is easy to increase the $K_a$ of DNA hybridization by increasing the number of base pairs.$^{21}$ Because the immobilized probe is a 15-mer, a 12-mer target strand with two additional complementary base pairs can be used to interrogate the same probe strands used to measure 10-mer hybridization. We measured $K_a$ and $\Gamma_{\text{max}}$ values for a 12-mer target intensity isotherm for two different substrates, where no correction for background fluorescence was required (see Supporting Information). The two additional base pairs on the 12-mer target yield an association constant, $K_{a12} = 1.5 \pm 0.1$ nM$^{-1}$, ~40× that of the 10-mer target, greatly lowering the solution concentration of target ssDNA needed to measure the roll-over in the isotherm. From the 12-mer $\Gamma_{\text{max}}$ values and slopes of the single-molecule 10-mer target isotherms measured at low concentrations on the same substrates (see Supporting Information), the 10-mer dissociation constant was determined to be $K_d = 38 \pm 3$ μM$^{-1}$, which is equivalent to values determined from the 10-mer target isotherm corrected for solution-phase fluorescence. Therefore, both methods provide consistent values of the 10-mer $K_a$ based on different approaches to measuring $\Gamma_{\text{max}}$.

Determining Dissociation Rates from Single-Molecule Residence Times. Although association equilibrium constants are important for characterizing the stability of DNA duplexes, knowledge of their binding and unbinding rates can provide insight into interactions that lead to formation and dissociation of these complexes and help in optimizing the parameters of hybridization assays. By tracking individual spots from frame-to-frame in videos (see example in Supporting Information and inset in Figure 3), residence times of bound target DNA on the surface can be measured, and the characteristic probe-target dsDNA dissociation rates can be determined from a histogram.
of cumulative residence times. The apparent dissociation kinetics measured using a single fluorescent label on the target molecule can be influenced by photobleaching and photo-blinking. Fluorophore photobleaching or formation of a long-lived triplet state can mimic an unbinding event because these processes result in disappearance of a fluorescent spot. The impact of photobleaching was reduced by querying locations of bound ssDNA for 2 s after apparent unbinding to see whether fluorescence returns to the same diffraction-limited spot and by bridging such blinking events. We tested the influence of photobleaching on the observed kinetics by determining $k_{\text{off}}$ with increasing illumination exposure time during intermittent imaging (see Supporting Information). At excitation power densities used to image reversible hybridization, photobleaching should increase the observed dissociation rates by <5%.

Residence time histograms were fit well ($R^2 > 0.99$) with a double-exponential decay with time constants $\tau_{\text{off1}}$ and $\tau_{\text{off2}}$

$N_{\text{res}} = A_1e^{(-t/\tau_{\text{off1}})} + A_2e^{(-t/\tau_{\text{off2}})}$

(3)

where $A_1$ and $A_2$ represent the corresponding number of events, and $N_{\text{res}}$ represents the number of bound molecules still residing after the time of binding $t$. An example fit of eq 3 to a residence-time histogram from the 10-mer target on the 250 μM probe immobilization surface is plotted in Figure 3. Double-stranded DNA dissociation with multieponential lifetime behavior has been reported previously, likely due to multiple modes or intermediates in association and subsequent dissociation. The double-exponential fits to the cumulative residence time histograms indicate that 85% of the duplexes resident on the surface at equilibrium dissociate with a $\tau_{\text{off1}} = 27$ s and 15% with $\tau_{\text{off2}} = 6$ s. The longer residence times of the dominant population suggest that they represent dissociation of the full probe-target double helix. The surface population weighted average dissociation time was $\tau_{\text{off}} = 23.3(\pm 0.5)$ s, corresponding to an average dissociation rate constant of $k_{\text{off}} = 1/\tau_{\text{off}} = 4.3 (\pm 0.1) \times 10^{-2}$ s$^{-1}$. With knowledge of $K_a$ and $k_{\text{off}}$, the association rate constant $k_{\text{on}}$ can be determined

$$k_{\text{on}} = k_{\text{off}}K_a$$

(4)

where $k_{\text{on}} = 1.64 (\pm 0.06) \times 10^6$ M$^{-1}$ s$^{-1}$, which is more than 3 orders of magnitude slower than a diffusion-limited association rate constant. This $k_{\text{on}}$ is similar to values determined by other researchers using single-molecule imaging to measure hybridization rates to immobilized probes ($k_{\text{on}} \sim 2.2 \times 10^6$ M$^{-1}$ s$^{-1}$ from Jungman et al. and $k_{\text{on}} = 2.1 (\pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$ from Dupuis et al.) and is within the range of previously measured rates of DNA association in free solution.

**Influence of Probe DNA Surface Density on Hybridization $K_a$, $k_{\text{on}}$, and $k_{\text{off}}$.** Previous studies of DNA hybridization on microarrays have shown that duplex stability is sensitive to probe density. Hybridization yield, association rate, and duplex stability all begin to decrease when average probe separation distances are less than ~10 nm, probably due to a combination of steric crowding and electrostatic repulsion between DNA phosphate backbones. In the present work, we have measured association equilibrium and rate constants for target hybridization to probes with spacing ranging from 60 to 320 nm, this range being between 6 and 30× more than the reported threshold for crowding. As expected, there is no trend in $K_a$ with probe surface density as shown in Figure 4A.

**Figure 4.** Interfacial hybridization equilibria and kinetics versus probe density. (A) Association constant, $K_a$ (B) dissociation rate constant, $k_{\text{off}}$, and (C) association rate constant, $k_{\text{on}}$ (black squares), plotted against probe ssDNA site density.

Surface probe density could influence the measured dissociation kinetics even at probe distances beyond the threshold for steric crowding or electrostatic repulsion. This influence would occur if a duplex dissociates and the liberated target ssDNA is captured by a neighboring probe strand before it can leave the interface. Because distances between neighboring probe strands are smaller than the optical point spread function, multiple hybridization-dissociation-recapture events could merge into an apparently longer event. If this were the case, the measured dissociation rate would decrease with increasing probe density, as the probability of nearby strand recapture increases. This process could be significantly enhanced if the dissociated target DNA adsors to the surface, thereby increasing the probability of surface diffusion-to-capture by a neighboring probe strand. We observe no trend in dissociation rate with probe density, as shown in Figure 4B, indicating that recapture of dissociated target ssDNA by nearby probe sites (within the point spread function) does not contribute significantly to longer apparent dissociation times.

Surface diffusion of an adsorbed target ssDNA population could also lead to recapture at more distant probe sites and play a role in the hybridization on-rate. If a dissociated target strand adsors to the surface and diffuses longer distances (more than the point spread function) before desorption or recapture, then the apparent association rate would increase with surface probe density because adsorbed ssDNA would be more likely to encounter a probe strand and rehybridize before desorbing. As shown in Figure 4C, there is no trend in the association rate constant with probe density, which suggests that surface diffusion and recapture of dissociated target DNA by more distant probe strands also play an insignificant role in the hybridization kinetics on these surfaces.
Although the lack of a probe-density dependence on the association and dissociation rates rules out significant recapture of dissociated target DNA, this evidence does not exclude the presence of a mobile population of adsorbed target DNA that could provide another path to hybridization besides target encounters from free solution. To test whether there are weak nonspecific surface interactions that may increase the interfacial concentration of target ssDNA, we investigated ssDNA nonspecific adsorption on blank sulfonate-passivated surfaces without immobilized probe strands. To detect a short-lived adsorbed target ssDNA population, we imaged blank surfaces groups at a fast (100 Hz) framing rate with a 25-fold higher excitation power density than used in single-molecule imaging and with a 500 pM target ssDNA concentration. In these videos (see example in Supporting Information), no DNA molecules could be detected where surface diffusion could be imaged and tracked; a small population of stationary target ssDNA molecules is observed on the surface, consistent with the results in Figure 1A. The fluorescence from target ssDNA on blank surfaces exhibits measurable higher average intensity when compared to interfacial images of buffer solution alone. This diffuse fluorescence intensity can be used to estimate the molecular population at the interface by comparison to the intensity of individual, stationary DNA molecules (see Supporting Information). From these results, the target ssDNA (number per unit area) at the interface was found to be \( \Gamma = 3.9 \pm 0.3 \times 10^6 \text{ cm}^{-2} \), which is indistinguishable from the predicted target ssDNA population within an evanescent wave depth of a 500 pM solution (see Supporting Information). The diffuse interfacial fluorescence from the target ssDNA solution at a sulfonate surface, therefore, could be entirely explained by solution-phase molecules in the evanescent wave. The uncertainty in the results sets an upper 95% confidence bound on the mobile adsorbed population of \( \Gamma_{\text{ssDNA}} = 3.6 \times 10^6 \text{ cm}^{-2} \), or \( \sim 9% \) of the population in the evanescent wave. Although collisions with probe strands are likely dominated by solution-phase target ssDNA, a small, mobile adsorbed population may be contributing to the observed association rate.

**Impact of Surface Tethering on Probe-Target Hybridization Kinetics.** The results above have shown that, at sufficiently low probe coverages, hybridization of target ssDNA is insensitive to probe density so that the kinetics represent independent binding events. With well-passivated surfaces, the adsorbed population of target DNA is small, and probe DNA should primarily experience collisions with solution-phase target molecules. Even with a passivated surface, however, other aspects of immobilizing the probe molecule on the surface, such as its tethering and proximity to the surface, may alter hybridization equilibria and kinetics relative to hybridization in free solution. For example, electrostatic interactions have been shown to influence the stability of surface-tethered duplexes.\(^{39,60}\) In addition, both hydrophobicity of the surrounding surface and reduction in conformational freedom of tethered ssDNA\(^{51}\) favor single-strand hairpin formation and may also influence duplex stability.

To examine the impact of probe immobilization on hybridization, we also measured hybridization kinetics in free solution of an un tethered probe-target pair having the same base sequences. To accomplish this goal, we take advantage of the environment-sensitive quantum yield of Cy3 used to label the target ssDNA. The quantum yield of Cy3 represents a competition between fluorescence emission and photoisomerization to a nonemissive state.\(^ {62}\) Photoisomerization is sensitive to local environment, where increased viscosity or steric hindrance can slow its rate and increase the fluorescence quantum yield.\(^ {63}\) Cy3 has been shown to associate with individual nucleotides and single- and double-stranded DNA,\(^ {40,65}\) and these interactions can influence the photoisomerization rate. Changes in fluorescence yield for Cy3 covalently attached to ssDNA are reported to be sensitive to duplex state, sequence,\(^ {66,67}\) and the attached nucleotide.\(^ {68}\)

We exploit the Cy3 fluorescence environment sensitivity to monitor hybridization in situ by detecting time-dependent changes in target Cy3 fluorescence upon addition of probe DNA. The interactions between Cy3 and the DNA structure, which provide a means of detection, could also influence duplex formation.\(^ {40}\) However, because the DNA sequences and dye labeling of the target strand are the same for both the immobilized probe and free solution reactions, the influence of the dye label should be equivalent, allowing the results to be compared. Fluorescence-emission time traces and spectra of Cy3-target DNA upon addition of 100 nM complementary probe ssDNA and 100 nM noncomplementary scrambled probe ssDNA are reported in the Supporting Information. These results show that, with the addition of excess complementary probe, hybridization causes a fluorescence increase from the target Cy3 of \( \sim 10\% \) over \( \sim 25 \text{ s} \), whereas addition of a scrambled sequence produces no measurable time-dependent change in fluorescence intensity. The association constant of the solution-phase duplex was determined by fitting the steady-state fluorescence intensity after sequential probe additions to an equilibrium association model (see Supporting Information)

\[
I_{eq} = \frac{(I_{0} - I_{ss})K_{[DNA_p]}}{1 + K_{[DNA_p]}} + I_{ss}
\]

where \( I_{eq} \) is the equilibrium target fluorescence intensity measured after each probe addition, \( I_{0} \) is the fluorescence intensity of the probe-target duplex, and \( I_{ss} \) is the intensity of the single-stranded target measured before probe additions. Fluorescence intensities and probe concentrations were corrected for dilution and probe strand depletion from hybridization, as described in the Experimental Section. The measured fluorescence intensity changes (average of 3 trials) are plotted versus the probe DNA concentration in Figure 5. The solution-phase association constant from a fit of eq 5 to the

![Figure 5](image) Free solution fluorescence target-probe association isotherm (black squares) with best fit from eq 5 (red line). Data points are the average of three trials.
data is $K_a = 31 (\pm 2) \mu M^{-1}$, which is remarkably similar to the average $K_a = 38 (\pm 1) \mu M^{-1}$ for the surface-bound probe.

The transient target-fluorescence response, $I_n(t)$, for the $n$th probe addition, are fit to the first-order relaxation kinetic model:

$$I_n(t) = (I_{eq,n} - I_{eq,n-1})[1 - \exp(-k'_n t)] + I_{eq,n-1}$$

where $I_{eq,n}$ is the equilibrium target fluorescence intensity for probe addition $n$, $I_{eq,n-1}$ is the intensity for the previous probe addition, and $k'_n$, the relaxation rate, is the sum of the on- and off- rates:

$$k'_n = [DNA_{p,n}]k_{on} + k_{off}$$  \hspace{1cm} (7)

An example fluorescence time trace is shown in Figure 6A with a fit to eq 6. Relaxation rates $k'_n$ averaged from three trials are plotted in Figure 6B, which vary linearly with the concentration of probe DNA as predicted by eq 7. From the linear fit of these results, the slope is the hybridization rate constant, $k_{on} = 1.41 (\pm 0.08) \times 10^6 M^{-1} s^{-1}$, and the intercept is the dissociation rate constant, $k_{off} = 4.6 (\pm 0.4) \times 10^{-2} s^{-1}$. The association equilibrium constant calculated from rate constants, $K_a = k_{off}/k_{on} = 31 (\pm 3) \mu M^{-1}$, agrees with the $K_a = 31 (\pm 2) \mu M^{-1}$ from equilibrium fluorescence intensities (Figure 5) and provides further support for the model.

The equilibrium and rate constants for free solution and immobilized probe hybridization are compared in Table 1. There is close agreement between rate constants for the two hybridization reactions. The dissociation rate constant of the surface-immobilized probe-strand should decrease its collision frequency with target probe-strand, indicating that the nearby surface has little impact on the duplex lifetime. The influence of probe strand immobilization is likely moderated by a surrounding passivated surface that reduces duplex-surface interactions, an adequate distance between probe strands that prevents interactions between them, and a high ionic strength (250 mM) that screens electrostatic repulsion with the underlying negatively charged surface.

Association rates of surface-bound probes would be expected to be slower than probes in free solution due to their restricted motion, which reduces collision frequencies compared to a reaction in free solution where both probe and target are freely diffusing. Rather than a slower rate, however, we observe a slightly (16%) faster association rate constant for immobilized probes compared to free solution. Measurements of fluorescence from target DNA at the passivated surface (see above) indicate that the adsorbed target DNA population is small, less than 10% of the solution population in the evanescent wave. A small mobile adsorbed population, however, could be contributing to $k_{off}$ if the hybridization efficiency of their collisions with probe strands were greater than those arriving from free solution. Increased efficiency in hybridization of surface-bound probes with target-ssDNA, either adsorbed or arriving from free solution, may arise from differences in the conformation of the probe strand at the interface compared to free solution. The tethered and excluded volume near the surface may limit the probe strand to a subset of conformations that are favorable for efficient duplex formation.

### CONCLUSIONS

We have developed a single-molecule fluorescence assay that can quantify equilibria and rates of hybridization between fluorescently labeled target ssDNA in solution and unlabeled probe DNA bound to a surface. Because of the low nonspecific adsorption and corresponding high selectivity of these surfaces, hybridization assays are possible with single-channel detection of only the labeled target-DNA, where higher surface densities of unlabeled probe ssDNA also increase the capture sensitivity and selectivity. Because the probe DNA strands are unlabeled, these substrates can be used for long periods of time and even reused in multiple experiments; we have examined the same substrate in the same excitation area for as long as 12 h with no increase in background or loss of response.

Hybridization kinetics on these surfaces were evaluated in what we believe is the first direct comparison of reversible DNA hybridization rates at a surface versus the same reaction in free solution. The dissociation rate of the surface immobilized probe-target duplex was equivalent to the dissociation rate of the same probe-target duplex in free solution, indicating that immobilization on these passivated surfaces has little impact on dsDNA duplex lifetime or stability. The association rate constants at the interface and in free solution were also comparable, which was surprising because immobilizing the probe strand should decrease its collision frequency with target molecules. More favorable conformations of the probe strand at the interface or a small mobile population of adsorbed target.

| Table 1. Hybridization of Immobilized and Free Solution ssDNA Probe with 10-mer Target |
|---------------------------------|----------|----------|----------|
| $K_a (\mu M^{-1})$ | $k_{on} \times 10^6 (M^{-1} s^{-1})$ | $k_{off} (s^{-1})$ |
| immobilized probe | 38 ± 1 | 1.64 ± 0.06 | 0.043 ± 0.001 |
| free solution probe | 31 ± 2 | 1.41 ± 0.08 | 0.046 ± 0.004 |
DNA could be contributing to the rate of association at the surface.

This simple method of detecting interfacial DNA hybridization at the single-molecule level opens up several new measurement opportunities. It provides a possible means to measure dissociation kinetics that are otherwise difficult to measure in free solution or with labeled probe sites at a surface. The high density of unlabeled probe sites employed in this work would allow investigation of oligonucleotide interactions with weak dissociation constants, where measurable populations of target molecules could be captured from solution concentrations far below $K_a^{-1}$. The quantitation limit of this experiment, given by $10 \times$ the blank standard deviation, is $\Gamma_{\text{min}} \approx 32$ molecules/FOV. This criterion could be met by a hybridization reaction with an association constant as low as $K_a = 65 \text{mM}^{-1}$, generating a quantifiable duplex population from a 1 nM solution target concentration. This concentration is $1/15,000$ the $K_a$ and sufficiently low so that fluorescence from the evanescent wave population would not interfere with detecting single-molecule hybridization events. Measurements of hybridization equilibria and dissociation rates are relevant to oligonucleotide systems that fall into this lower $K_a$ range, including split aptamers and shorter oligonucleotides with single nucleotide polymorphisms (SNPs).[7,9]

The combination of a high probe-site density and single-molecule counting of bound duplexes of a sample yields a potentially sensitive measurement of target molecules in solution. From the quantitation limit for counting bound duplexes, $\Gamma_{\text{min}}$, above the limit for detecting target DNA in solution can be estimated by rearranging eq 2

$$[\text{DNA}^+]_{\text{min}} = \Gamma_{\text{min}} / K_a \Gamma_{\text{max}}$$

This relationship shows the benefit of utilizing a higher probe site density, $\Gamma_{\text{max}}$, which reduces the target DNA concentration required to produce the minimum number of bound duplexes needed for quantification, $\Gamma_{\text{min}}$. The solution-phase limit is also proportional to $K_a^{-1}$, so that longer, more tightly bound duplexes will produce lower detection limits. For example, the association constant of a 12-mer complementary target ssDNA used for quantifying probe site densities (see Supporting Information) is $K_a = 1.5 \times (0.1) \text{mM}^{-1}$, or 40X greater than the 10-mer target. On the basis of its dissociation constant, the solution concentration that would produce a quantifiable number of 12-mer duplexes, $\Gamma_{\text{min}}$ is quite small, $[\text{DNA}^+]_{\text{min}} = 50 \text{fM}$ versus 2 pM for a 10-mer duplex. The slower dissociation rate of the more stable 12-mer duplex, however, requires a much longer time (~30 min) to reach equilibrium compared to less than a minute for a 10-mer target.

This point raises a challenge of measuring longer, more tightly bound duplexes to lower the detection limits, namely, that these will exhibit a very slow approach to equilibrium due to slow dissociation rates. Under these circumstances, instead of an equilibrium measurement, a dosimetry assay could be performed by measuring the rate of target accumulation on high-density surfaces. Using the $k_{\text{off}}$ rate for 10-mer hybridization (on-rates have been shown to be insensitive to strand length), one can predict the rate of binding of 5 fM target ssDNA to a high density capture surface: $\Gamma_{\text{max}} k_{\text{on}}[\text{DNA}^+] A = 0.22$ molecules per FOV area (A) per minute. On the basis of this rate, incubation for 2.5 h with 5 fM target ssDNA would generate a reliably detectable $\Gamma_{\text{min}} = 32$ molecules. Although longer DNA-duplex strands in such an assay could be quite long-lived, their removal could be readily achieved by melting the duplex at higher temperatures, thus renewing the surface. Reversible hybridization of much longer ssDNA targets could also be achieved by carrying out measurements at elevated temperatures closer to the melting transition of the target-probe duplex. Although this decreases $K_a$ and therefore lowers the detection sensitivity, the speed of measurement increases, and characterizing the hybridization kinetics of longer duplexes is relevant to understanding the sequence selectivity needed for diagnostic applications.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03832.

Additional information is presented on chemical sources and structures, the DNA immobilization scheme, image analysis, measuring probe coverage with longer DNA targets, background correction, residence time measurements, testing the influence of photobleaching, high-speed imaging to detect adsorbed target DNA, free solution hybridization measurements, and derivation of free solution hybridization equilibria (PDF)

An example video shows reversible hybridization of 150-pM ssDNA to probe ssDNA on a passivated glass surface (AVI)

A second video is a control, where 500-pM ssDNA is imaged at a blank, sulfonate-passivated surface (AVI)

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported in part by the National Science Foundation under Grant CHE-1306204.

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