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Oligonucleotide Dynamics and Hybridization at Solid-Liquid Interfaces

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Oligonucleotide Dynamics and Hybridization at Solid-Liquid Interfaces

By Jon H. Monserud

B.S., Colorado School of Mines. 2009
This thesis entitled:
‘Oligonucleotide Dynamics and Hybridization at Solid-Liquid Interfaces’
written by Jon Hansen Monserud
has been approved for the Department of Chemical and Biological Engineering

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Abstract

Monsrud, Jon Hansen (PhD, Chemical and Biological Engineering Department)

Oligonucleotide Dynamics and Hybridization at Solid-Liquid Interfaces

Thesis directed by Professor Daniel K. Schwartz

Biosensors, and other diagnostic techniques, are dependent on the specific interactions of nucleotides on a functionalized surface. Here, we study oligonucleotide dynamics and hybridization at solid-liquid interfaces. In particular we are interested in understanding how oligonucleotides, behave in various solid-liquid interfacial environments. First, we prepared substrates with varying hydrophobicity to explore the effects of molecular size and surface hydrophobicity on oligonucleotide interfacial dynamics. Next we studied the mechanisms of surface-mediated oligonucleotide hybridization. This study cultivated an interest in the search strategies of interfacial reactions. The majority of this field is dominated by theoretical and ecological studies. We felt that with our technique we could further the understanding of these fields. This led us to our final study exploring the effects of hydrophobicity on the interfacial search strategies of oligonucleotides.

The findings presented here provided further understanding of oligonucleotide interfacial behavior that may be used to enhance biosensor and other nucleotide based technologies. For example, studies at the solid-liquid interface revealed that surface residence time decreased with increasing ssDNA length on hydrophobic surfaces, particularly for longer oligonucleotide chains. Similarly, the interfacial mobility of polynucleotides slowed with increasing chain length on hydrophilic, but became faster, on hydrophobic surfaces. These combined observations suggest that long oligonucleotides adopt conformations minimizing hydrophobic interactions. Furthermore, when
pathways of DNA hybridization were explored, the vast majority of molecules from solution adsorbed non-specifically (without directly hybridizing) to the surface, where a brief 2-dimensional search was performed with a 7% chance of hybridization. We observed that hybridization was reversible, and had two distinct modes of melting (i.e. de-hybridization) corresponding to long-lived (~15s) and short-lived (~1.4s) hybridized time intervals. Finally, we designed experiments to probe the search behavior of oligonucleotides as a function of surface hydrophobicity. This study demonstrates that oligonucleotides adopt alternating Lévy-flight and Brownian search behavior regardless of surface hydrophobicity. This search strategy was enhanced in the hydrophobic environment, however, duplex DNA had shorter hybridized time intervals. These studies advance the understanding of oligonucleotide interfacial dynamics and provide examples of surface modifications that can influence hybridization stability and molecular search efficiency. Thus we have used a comprehensive study of oligonucleotides to gain a better understanding of oligonucleotide dynamics and hybridization at solid-liquid interfaces.
This work is dedicated to my parents, Jon and Karen Monserud,
for giving me the chance to prove and improve myself in all my walks of life

This work is also dedicated to my loving wife, Hailey E. Monserud

“Always”
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Chapter 1: Introduction

1.1 Nucleic Acid Structure

Deoxyribonucleic Acid (DNA) is arguably one of the most important biomacromolecules. This is attributed to the fact that the DNA molecule contains the genetic information employed by cells to make various cellular components, RNA molecules and proteins.\(^1\)\(^2\) Polynucleotide chains of DNA are composed of four organic bases: adenine (A), guanine (G), thymine (T), and cytosine (C) (Figure 1.1). The four bases are attached to a 2-deoxyribose molecule. This molecule constitutes the nucleotide monomer. There are a total of four nucleotides which coincide with the four organic bases. These bases preferentially hydrogen bond in pairs, adenine to thymine and guanine to cytosine (Figure 1.1). The triphosphate form of the nucleotides can be linked through various reactions to form phosphodiester bonds between two adjacent deoxyribose groups. This reaction, under certain conditions will polymerize to form single stranded DNA (ssDNA).

![Figure 1.1 - Watson-Crick Base pairing scheme. R group represents attachment to deoxyribose group. Note: Image rendered in ACD/Chemsketch freeware version.](image-url)

\(^1\) Reference 1
\(^2\) Reference 2
Structurally, ssDNA can be broken down into two parts, the nucleobases and phosphodiester backbone (Figure 1.2). The chemistry of each of these DNA molecule components is unique and provides insight into how ssDNA will interact with a surface. The phosphodiester backbone contains a periodic phosphate anion (one per nucleotide at most pH values) which gives DNA an overall negative charge. This attribute results in electrostatic interactions with surfaces. DNA contains several Nitrogen and Oxygen containing functional groups, and thus, can form favorable Hydrogen bonds with surfaces. The phosphodiester backbone is more polar than the nucleobases due to the phosphate anions, and as such, is more hydrophilic than the nucleobases. This structural feature gives the DNA molecule an amphiphilic nature so it is expected that hydrophobic interactions will influence surface interactions. Due to these qualities ssDNA adsorption to a surface is dominated by the hydrophilicity of the surface, surface charge, and the capacity of a surface to form hydrogen bonds.

Figure 1.2 - Short chain single stranded oligonucleotide (3’-ATCG-5’) demonstrating amphiphilic nature of ssDNA created by different chemistries present in the phosphate backbone and nucleobase portion of the ssDNA
1.2 Adsorption of Nucleic Acids at The Solid-Liquid Interface

1.2.1 Electrostatic Effects

Electrostatic interactions are one of the primary phenomena that affect DNA adsorption to a surface. As discussed earlier, DNA has a charge density of one negatively charged phosphate group per nucleotide. Common surfaces such as charcoal or silica have an overall positive or negative charge. This combination suggests that electrostatic interactions between surfaces and DNA macromolecules will significantly affect adsorption of DNA to surfaces.

In the study performed by Saoudi et al., dsDNA adsorption behavior was observed on surfaces treated to either possess a positive or negative charge. These surfaces consisted of bare silica, polypyrrole (PPy), aminated silica, PPy modified silica, PPy modified silica functionalized with carboxylic acids, and PPy modified silica that has been aminated. The amount of DNA adsorbed per mass of substrate (b) was evaluated using the following equation:

\[ b = (a_0 - a) \frac{vc}{m} \]  

(1.1)

Where \( a_0 \) and \( a \) are the absorbance of light at 260 nm of the total amount of DNA added and the absorbance of 260nm light of the equilibrium DNA, respectively, \( v \) is the total solution volume of the solution as described, \( c \) is the mass of substrate is \( m \) and \( c \) is a constant relating absorbance to DNA concentration (50 \( \mu \)g dsDNA per ml at 260 nm).
It was observed, that the negatively charged silica surface exhibited no DNA adsorption in the absence of electrolyte or solutions with low ionic strength (Figure 1.3) which was confirmed by other authors.\(^4\), \(^5\), \(^6\) It is agreed upon that this phenomenon is attributed to the electrostatic repulsion between the negatively charged surface and the negatively charged DNA phosphodiester backbone. This phenomenon suggests that in general, negatively charged

![DNA adsorption isotherms](image)

Figure 1.3 - DNA adsorption isotherms onto PPyCl powder (●), silica (□), PPy–silica (△), silica–NH₂ (⋆), PPy–silica–NH₂ (■), and PPy–COOH–silica (●). The conditions were 0.10 \(M\) sodium phosphate buffer (pH 7) at 20°C. Adsorbed amounts are expressed in mg/g rather than mg/m² to avoid incurring errors due to uncertainties in the effective surface areas of the nanocomposites.\(^6\)
surfaces will have reduced adsorption of DNA. Similarly, it is noted that formal positive charges on a surface will increase adsorption of the negatively charged DNA. This is attributed to the attractive electrostatic interactions between the positively charged surface and the negatively charged biomolecule. However, it was noted that at pH 7 the aminated PPy-silica surface has an overall neutral charge indicating that the surface silinanol groups are balanced by the protonated amine groups on the surface. Despite the fact that only a neutral surface charge, is observed, the DNA adsorption is ~15 times greater than the untreated PPy-silica which is similar to bare silica with regard to electrostatics, therefore it is unnecessary for the surface to be positively charged. This suggests that the attractive interactions between the isolated –NH$_3^+$ groups on the surface and DNA chains are sufficient to induce/enhance DNA adsorption.

In a study performed by Gani et al.$^5$ the effect of neutral salts in solution on DNA adsorption is explored by evaluating the adsorption isotherm on charcoal of B-Form DNA in the presence of NaCl at varying ionic strengths with constant pH and temperature. This study involved an approach similar to the one used by Saoudi et al and is described in detail in the hydrophobicity section of this review. This analysis demonstrated that as the ionic strength of the solution increases, the maximum adsorption of DNA increases (Figure 1.4). For this system the initial slope of the isotherms was analyzed to assess the interaction energy of adsorbate with adsorbent. As the ionic strength increased from $\mu = 0.01$-0.10 the initial slope increased from 2.18-2.98.$^5$ This increase correlates with a decrease in Gibbs free energy of adsorption and suggests that increasing ionic strength decreases the double-layer potential, which acts as an energy barrier to adsorption. This trend can also be attributed to the fact that in high ionic
strengths DNA compacts, which will allow for greater number of DNA molecules per surface area. This trend is found to be consistent with negatively charged surfaces as well. The proposed theory for negatively charged surfaces is that at higher ionic strengths the negative potential of the DNA and the surface are reduced, thereby, lowering the electrostatic penalty for placing negatively charged DNA molecules next to the negative surface.

As described earlier, interesting electrostatic phenomenon occur with DNA adsorption onto surfaces in the presence of neutral inorganic salts. In the study by Cárdenas et al. the

![Figure 1.4 - Plot of $\Gamma_{2}^{-1}$ against $X_2$ for the adsorption of native DNA on the surface of charcoal at pH 6.5 in the presence of NaCl: (O) 28 °C, $\mu = 0.01$; (--) 28 °C, $\mu = 0.05$; (Δ) 28 °C, $\mu = 0.10$; (■) 18 °C, $\mu = 0.05$; (▲) 37 °C, $\mu = 0.05$.](image-url)
effects of cationic surfactant (CTAB) concentration on DNA adsorption behavior on negatively charge silica surfaces. In this study, a combination of Ellipsometry to probe DNA adsorption to silica, and Surface force experiments (using an interferometric surface force apparatus) were used to probe the conformation of DNA adsorbed to the surface. In the ellipsometric evaluation of surface adsorption, an isotropic homogenous four layer model (three interface) was employed to extract the mean refractive index ($n_f$) and thickness ($d_f$) of the adsorbed layer. Using this information, combined with the refractive index of the solution ($n_0$) and the incremental change in refractive index with respect to change in solution concentration ($dn/dc$), the adsorbed amount of DNA in mg/m$^2$ ($\Gamma$) can be evaluated with equation 1.2.

$$\Gamma = \frac{(n_f-n_0)d_f}{dn/dc} \quad (1.2)$$

Using this equation the concentration of CTAB was varied for a constant DNA concentration and the Adsorption isotherm was evaluated (Figure 1.5). In this study it was observed that an excess amount of CTAB was required to induce adsorption with the lowest concentration to induce adsorption ($6 \times 10^{-4}$ M CTAB) corresponded to a molar charge ratio of $\rho$ of 0.31. \footnote{7} This indicates that for every negative phosphate group on the DNA backbone there were ~3 positively charge CTAB molecules. The largest observable point was that at which turbidity was observed prior to phase separation. At this concentration, for every negative phosphate group on the DNA backbone there were ~4 positively charge CTAB molecules (molar charge ratio of $\rho$ of 0.26). This observation suggests a limitation to surfactant based adsorption systems. However the region of turbidity is below the critical micelle concentration which suggests that the DNA and cationic surfactant are complexing which results in the charge shielding that
enhances adsorption on to negatively charged substrate. As discussed earlier, adsorption is favored once the charge density both the DNA and the surface has been sufficiently decreased.

As expected, DNA adsorption to surfaces is heavily influenced by the electrostatic interactions between the negatively charge DNA macromolecules and the formal charge on the surface. Therefore, it was observed that adsorption to negatively charged surfaces was reduced or eliminated due to the repulsive electrostatic interactions, while positive surfaces exhibit attractive electrostatics resulting in an increase in DNA adsorption. When neutral salts are dissolved in the DNA solutions electrostatic shielding of the surface and DNA occurs which

![Figure 1.5 - Plot of $\Gamma^1$ Adsorption isotherm for C$_{12}$TAB (filled circles) and DNA-C$_{12}$TAB mixtures (open circles) as measured by ellipsometry. The 0.06 mg/mL DNA was used for the mixed system. Lines are included as guides for the eye.]

7
allows for greater surface adsorption of DNA. Lastly, it was observed that in cationic surfactant systems below the critical micelle concentration DNA-surfactant complexes form which enhance DNA surface adsorption. Overall it was observed through various studies that the electrostatic phenomena observed with DNA significantly impact adsorption.

1.2.2 Hydrophobicity Effects

The process of adsorption involves the approach of a molecule to a surface where it may interact favorably and adsorb. Hydration can interfere with this mechanism by via water bound to adsorption sites requiring the release of water prior to adsorption. Thus, hydrophobicity of the surface and molecule play an important role as they define the quantity of bound water to both molecule and surface.

Gani et al. have extensively explored adsorption of DNA on different solid powders. In this study, adsorption of DNA was measured as a function of mole fraction of nucleotide at a fixed ionic strength, pH, and type of DNA. For this study a series of DNA samples were prepared at a known concentration (C\text{t}) and known volume (V). The solutions would then have a known weight of solid (W) with a specific surface area per gram (A). The samples were allowed to reach equilibrium conditions over the course of 20 hours with mild shaking. The solution could then be extracted, after the solid settled, and the DNA concentration in solution (C_2) evaluated via spectrophotometer and calibration curve. Then, using the following equation the surface coverage could be calculated:
\[
\Gamma_2 = \frac{(C_2 - C_1)}{W_A} \times 1000
\]  \hspace{1cm} (1.3)

As expected, it was observed that as the mole fraction of the nucleotide is increased while pH, temperature, and ionic strength are held constant, the surface coverage increases until a fixed maximum is observed, this corresponds to the formation of a monolayer of DNA\(^5\) at a critical mole fraction of DNA in solution (Figure 1.6). As seen in Figure 1.6, many of the surfaces exhibit further adsorption past the critical mole fraction discussed earlier, this observation was attributed to the formation of DNA multilayers. The \(\Gamma_2^m\) values which correspond to the critical mole fraction of DNA, decrease in the following order chromium>charcoal>silica>Cl\(^-\) resin>alumina>sephadex.

Figure 1.6 - Plot of \(\Gamma_2^1\) against \(X_2\) for the adsorption of native DNA on different solid surfaces at 28 °C, pH 6.5, and ionic strength 0.05 in the presence of NaCl: (△) silica; (△) chromium; (●) sephadex; (▲) Cl\(^-\) resin; (☐) charcoal; (●) alumina (25 °C).\(^5\)
The distinct trend in $\Gamma_2^m$ values versus surface did not demonstrate significant electrostatic effects as discussed by Melzak et al.\textsuperscript{4} which was partially attributed to the presence of electrolyte shielding of the DNA. More interestingly the authors explored the hydrophobicity of the various surfaces. This was done through analysis of the amount of water adsorbed to the surface at 95% humidity. It was observed that the moles of water absorbed per kilogram of solid decreased in the following trend alumina$>$Cl$^-$ resin$>$silica$>$sephadex$>$charcoal$>$chromium which is almost the opposite of the $\Gamma_2^m$ trend (chromium$>$charcoal$>$silica$>$Cl$^-$ resin$>$alumina$>$sephadex). Based on these observations, it was concluded that the hydrophobicity of the surface plays a large role in DNA adsorption to the surface. Effectively more hydrophilic surfaces adsorb more water which competes with DNA for surface sites.

In order to assess the affects of the hydrophobicity of DNA itself Melzak et al.\textsuperscript{4} several conformations of DNA (B-form, supercoiled plasmid, and linearized plasmid) were studied while adsorbing to silica surfaces, using methods similar to those used by Gani et al discussed earlier. In this study it was discussed how various conformations of DNA binds water. More specifically there are $\sim$19.3 water molecules per nucleotide in B-form DNA.\textsuperscript{4} The bound water works to hamper the adsorption of DNA by interfering with favorable interactions between the surface and DNA (\textit{i.e.} Hydrogen bonding). In the study sodium perchlorate was used as the neutral salt. Each perchlorate anion is known to bind $\sim$4 water molecules, therefore, as the concentration of sodium perchlorate in solution increases there is less free water to solvate the DNA, as such, when the perchlorate concentration is increased past the concentration necessary to induce
adsorption, $\Gamma_2^m$ increases. This observation is attributed to a Le Chatelier's principle-like effect where free water is bound by perchlorate anions so the equilibrium is shifted toward DNA-silica complexing.

In another study by Gani et al.\textsuperscript{8} the effects of changing the type of salt dissolved in solution (NaCl + CaCl\textsubscript{2}, NaCl + AlCl\textsubscript{3}, NaCl) would have on adsorption of DNA to casein surfaces were examined. Changing the type of salt in solution would alter the electrostatic interactions between DNA and the surface. For example multivalent cations have the ability to bind to phosphate groups and make an ion pair which would reduce the charge density of the molecules as well as the double layer potential.\textsuperscript{5} In this study, the authors used the photospectroscopic techniques combined with equation 1.3 and a calibration curve to assess the adsorption of DNA (Figure 1.7). It was noted that unmodified DNA is unable to adsorb to the casein surface in the presence of only NaCl (not shown).\textsuperscript{6} In the case of casein powder, the surface is highly hydrophilic and has excessive bound water, as such, the NaCl is unable to efficiently dehydrate the surface to eliminate the energy barrier associated with displacement of surface bound water. The multivalent cationic systems, however, induce adsorption of DNA to the surface which is attributed to their ability to reduce the amount of free water in the system thereby exposing sites on the surface as well as on the DNA that may interact favorably. Thus it is observed that as the valancy of the cation increases so does DNA adsorption.
The mechanism associated with adsorption suggests that hydrophobicity of the surface, as well as that of the adsorbing molecule, will influence the efficiency of adsorption. It has been shown that in general, as the hydrophobicity of the surface increases so does the adsorption of DNA. This is attributed to the fact that the more hydrophobic a surface the less water bound to the surface needs to be displaced in order for DNA to interact.\(^5\) Hydrophobicity of DNA also affects the adsorption of DNA to a substrate since the water bound to the DNA must also be displaced.\(^5,4\) As such, the presence of various neutral salts such as sodium perchlorate have the ability to reduce the amount of free water in a system dehydrating the surface and DNA, which

![Plot of \( \Gamma_{2} \) against \( X_2 \) for the adsorption of DNA on the surface of casein at 28°C, pH 6.0, and \( \mu = 0.05 \) in the presence of salts: (--) NaCl+CaCl\(_2\), native DNA; (O) NaCl +AlCl\(_3\), native DNA; (●) NaCl, heat-denatured DNA; (△) NaCl + CaCl\(_2\), heat-denatured DNA.\(^8\)](image-url)
enhances adsorption. It was demonstrated that as the valancy of cations increases so does adsorption, which was attributed to the dehydrating effect described above. Therefore, it is apparent that the Adsorption of DNA is significantly affected by the hydrophobicity of both the molecule and the surface in question.

1.3 Self-Assembled Monolayer

Self-assembled monolayers (SAM) of organic molecules are molecular assemblies formed spontaneously on surfaces by adsorption and organization into more or less large ordered domains. In some cases, molecules that form the monolayer do not interact strongly with the substrate. In other cases the molecules possess a functional group that has a strong affinity to the substrate and anchors the molecule to it. These strong affinities are generally achieved with molecules consisting of a head, tail and a functional end group which may or may not be present. Common head groups include thiols, silanes, phosphonates, etc. while the tail is selected for desirable chemistries, i.e. long chain hydrocarbons for a hydrophobic interface.

SAMs are created by the adsorption of molecules onto a substrate from either a vapor or liquid phase. Adsorption of the molecules to the surface is followed by a slow organization of the adsorbed molecules facilitated by favorable interaction of head and tail groups. Initially, molecules are sparsely distributed on the surface forming nucleation sites for crystalline growth on the surface. In these low surface density regimes molecules generally form a disordered mass or sparsely distributed ordered two-dimensional regions. Surfaces exposed for longer
periods of time, minutes to hours, begin to form crystalline structures around the nucleation sites on the substrate surface. During this process the head groups assemble together on the substrate and as surface density increase the tail groups organize and point away from the substrate. These areas of close packed molecules will continue to grow until reaching other growing regions and the surface of the substrate is covered in a single monolayer. Adsorption of molecules is generally an energetically favorable process because adsorbing molecules lower the free-energy of the surface. In many cases the adsorbate molecules are stabilized by the strong chemisorption of the head groups. For example, thiol-metal bonds are fairly stable in a variety of temperatures, solvents, and potentials. Silane based head groups react with hydroxyl groups on a substrate to form stable covalent bonds. Due to its high stability and ease of use silane chemistry is used exclusively in the work describe here.

1.4 Ellipsometry

Ellipsometry is an optical technique that measures a change in polarization as light reflects or transmits from a material structure. The measured signal is the change in polarization as the incident radiation interacts with the material of interest. The polarization change is represented as an amplitude ratio, \( \Psi \), and the phase difference, \( \Delta \). These parameters make up the complex reflectance ratio, \( \rho \), and is the value measured by ellipsometry (equation 1.4).  

\[
\rho = \frac{r_P}{r_s} = \tan \Psi e^{i\Delta} \quad (1.4)
\]

The polarization state of the light incident upon the sample may be decomposed into an s and a p component. The s component is oscillating perpendicular to the plane of incidence and parallel to the sample surface, and the p component is oscillating parallel to the plane of
incidence (Figure 1.8a). The amplitudes of the s and p components, after reflection and normalized to their initial value, are denoted by $r_s$ and $r_p$, respectively. The initial polarization is set by a polarizer before the beam passes through a quarter wave plate to turn the linearly polarized light into elliptically polarized light (Figure 1.8b). The use of elliptically polarized light is where ellipsometry acquired its name. When the elliptically polarized light reflects off of a surface it is converted into linearly polarized light.\textsuperscript{18} The polarization of this light is measured by identifying the null point (polarizer orientation at minimum intensity) by a second polarizer (analyzer). For a given wavelength of light and angle of incidence it is possible to extract the

Figure 1.8 – (A) The polarization state of the light incident upon the sample (B) Reflection ellipsometry diagram
amplitude ratio, $\Psi$, and the phase difference, $\Delta$, from the analyzer (A) and polarizer (P) positions at the null point:

$$\Psi = A$$  \hspace{1cm} (1.5)

$$\Delta = 2P + \frac{\pi}{2}$$  \hspace{1cm} (1.6)

Physical parameters, such as film thickness, can’t be directly extracted from $\Psi$, and $\Delta$. So a multilayer model is developed that combines Fresnel coefficients, Snell’s law, and Film phase thickness for each layer of the model. Using Jones matrix formalism the jones matrix for an interface and layer can be derived. A scattering matrix can be derived via summation of interface and layer matrix, which is used to estimate the values of $r_s$ and $r_p$.\textsuperscript{18-20} The model is then fit to the experimental data by weighted non-linear regression (Levensburg-Marquardt Algorithm) to acquire an estimate of film thickness for layers with a known refractive index.\textsuperscript{20, 21}

In this work, reflection ellipsometry is used to estimate film thickness and evaluate the quality of deposited SAMs.

1.5 Contact Angle

The contact angle is an angle created by a liquid coming in contact with a solid material. It quantifies the wettability of a solid surface by a liquid \textit{via} the Young equation. This angle is determined by properties of both the solid and liquid and the interaction and repulsion forces between them and by the three phase interface properties (gas, liquid and solid).\textsuperscript{22} A system of solid, liquid, and vapor at a given temperature and pressure has a unique equilibrium contact
angle. The equilibrium contact angle reflects the relative strength of the liquid, solid, and vapor molecular interaction.

The sessile drop technique is a method used for the characterization of solid surface energies. The simplest way of measuring the contact angle is with a goniometer, which allows the user to do so visually. The droplet is deposited by a syringe pointed vertically down onto the sample surface, and a high resolution camera captures the image, which can then be analyzed using image analysis software.\(^{23}\)

In this work, the contact angle is used to probe hydrophobicity of an interface by water sessile drop experiments. In these experiments, if the water molecules are strongly attracted to the solid surface the water droplet will spread out on the surface giving lower contact angles, indicating that the surface has a more hydrophilic character. Therefore, relative hydrophobicity of a surface can be assessed *via* contact angle measurements.

1.6 Total Internal Reflection Fluorescence Microscopy

Various mechanisms are often employed in fluorescence microscopy applications to restrict the excitation and detection of fluorophores to a thin region of the specimen. Elimination of background fluorescence from outside the focal plane can dramatically improve the signal-to-noise ratio, and consequently, the spatial resolution of the features or events of interest. Total internal reflection microscopy (TIRFM) uses an induced evanescent wave to excite fluorophores in a limited volume near the interface between two media with different refractive indices. In the experiments in this work an excitation laser is shown through a prism at an angle so that
the beam is totally internally reflected off a solid-liquid interface (Figure 1.9). This causes an evanescent wave to propagate into the solution. The nature of this wave is that it decays exponentially away from the interface. Therefore, fluorophores can only be excited in the near surface region (100nm).超 This makes the technique ideal for monitoring interfacial dynamics of fluorescently labeled DNA at the solid-liquid interface.25-27

1.7 Resonance Energy Transfer

Spatial information in fluorescence imaging can be acquired via resonance energy transfer (RET). This technique involves the non-radiative energy transfer from a donor fluorophore to an acceptor fluorophore that occurs when the two fluorophores are within 1-10nm,28 where the specific distance-dependence is characteristic of the specific RET-pair. For RET to occur, the emission spectrum of the donor fluorophore must have significant overlap with the excitation spectrum of the acceptor fluorophore. The spectral overlap integral \( J \) with respect to wavelength \( (\lambda) \) can be calculated as

\[
J = \int f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad (1.7)
\]
where subscripts A and D represent acceptor and donor, respectively, $f$ is the normalized emission spectrum and $\epsilon$ the molar extinction coefficient. Using the spectral overlap integral along with the refractive index ($n$) of the medium, the donor fluorophore quantum yield ($Q_\text{o}$), the dipole orientation factor ($\kappa^2$) and Avagadro’s number ($N_A$), the Förster radius can be calculated as

$$R_0^6 = \frac{9 Q_\text{o} \ln 10 \kappa^2}{128 \pi^5 n^4 N_A}$$  \hspace{1cm} (1.8)

The Förster distance, is the distance where the non-radiative energy transfer between donor and acceptor fluorophores is 50%.\(^29\) The Förster for Alexa-488 and Quasar- 670 is approximately 7 nm, the exact value is dependent on the molecular orientation ($\kappa^2$) and medium conditions ($n$).\(^30\) The Förster radius coupled with the experimentally observed fluorescence intensity of the donor ($F_D$) and acceptor ($F_A$) can be used to calculate the separation of the RET pair

$$r = R_0 \left( \frac{F_A}{F_D} \right)^{1/6} \left( \frac{F_D}{F_A} \right)^{1/6}$$  \hspace{1cm} (1.9)

The ratio of acceptor fluorescence at zero separation and donor fluorescence at infinite separation is expected to be on the order of unity. Due to the uncertainty in this assumption, as well as those described above it is challenging to calculate an exact value for $r$ during RET experiments. Therefore, in this work the relative end-to-end distance ($d$) is used to characterize DNA hybridization events.\(^31\)

$$d = \left( \frac{F_D}{F_A} \right)^{1/6}$$  \hspace{1cm} (1.10)
1.8 **References**


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Chapter 2: Effects of Molecular Size and Surface Hydrophobicity on Oligonucleotide Interfacial Dynamics

2.1 Introduction

Biosensors and other diagnostic technologies often rely on the interactions of nucleic acids in the vicinity of interfaces. For example, deoxyribonucleic acid (DNA) microarray technology has rapidly become an indispensable tool for studies of genetic content because they permit the simultaneous multiplexed analysis of large numbers of nucleic acid fragments. Microarrays cover a wide range of applications including genotyping, detection of single nucleotide variations in a DNA sequence (single-nucleotide polymorphism), and analysis of gene expression networks. Recent single molecule sequencing-by-synthesis technologies represent a massive parallelization of the array approach, potentially permitting even higher data densities. DNA-based technologies typically require nucleotides or oligonucleotides (generally less than 100 bases long) to form Watson-Crick base-pairing complexes with surface-immobilized nucleic acids with a very high degree of specificity; concurrent non-specific adsorption results in background noise or errors. Therefore, surface coatings should encourage base-pairing by inducing molecular conformations that favor hybridization and by enabling adsorbed ssDNA to explore large regions of the surface (e.g. long residence time and high mobility). Recent theoretical work suggests that subtle details of surface mobility can have significant effects with respect to the efficiency of this “targeting” process. At the same time, these surfaces should be designed to inhibit irreversible non-specific DNA adsorption as much as possible. However, because DNA is structurally complex and can engage in many competing
non-covalent interactions with a surface, the optimal type of surface chemistry is not immediately apparent.

The largest contributors to DNA-surface interaction are hydrophobic interactions, hydrogen bonding (H-bonding), and van der Waals interactions. Figure 2.1 schematically illustrates some ways in which these interactions may influence dynamic behavior on surfaces. ssDNA is highly flexible with a statistical segment length of one monomer unit and has the ability to expose either the phosphodiester backbone or nucleobases to a surface. The nucleobases,

![Figure 2.1 - A schematic diagram of hypothetical surface interactions for the nucleobase cytosine (left molecule) and the phosphate backbone (right molecule). The surfaces represent (a) a non-hydrogen-bonding hydrophobic surface, and (b) a non-hydrogen-bonding hydrophilic surface. All chains in the surface layer are the same length; the “shorter” apparent chains are intended to demonstrate immersion/intercalation of the adsorbate within the monolayer.](image)
consisting of either one (pyrimidine) or two (purine) aromatic rings, are significantly more hydrophobic than the phosphate backbone and exhibit an affinity to non-polar environments.\textsuperscript{12} Thus, molecular conformations that minimize nucleobase exposure are preferred in aqueous solution. Similarly, exposed nucleobases are expected to interact relatively weakly with a non-H-bonding hydrated hydrophilic surface (Figure 2.1b) and more strongly with a hydrophobic surface (Figure 2.1a). The opposite is true for the phosphodiester backbone (Figures 2.1a,b).\textsuperscript{9,10}

Hydrophobic interactions are mediated by water and tend to cause hydrophobic molecules to cluster in aqueous conditions.\textsuperscript{13} These interactions are complex and manifest in different ways depending on the attributes (such as molecular size) of the model system.\textsuperscript{14} For large biomolecules, the thermodynamic aspects of the hydrophobic effect are critically important determinants of molecular conformation and stability.\textsuperscript{15} The hydrophobic effect also exhibits a unique, non-Arrhenius temperature dependence. In particular, hydrophobic interactions typically increase in strength up to a maximum at 293–303K as a result of increasing enthalpic contributions. Above 303K enhanced entropic contributions result in a decrease in the strength of hydrophobic interactions.\textsuperscript{14,16} Therefore, a distinctive anomalous non-monotonic temperature behavior is generally observed in systems dominated by hydrophobic interactions. For example, Haidacher \textit{et al.} observed non-monotonic temperature-dependence of the retention time of a model hydrophobic probe molecule using several hydrophobic interaction chromatography columns.\textsuperscript{15}
In this work, oligoethyleneglycol-modified fused silica (OEG), and n-octadecyltriethoxysilane-modified fused silica (OTES) were used to probe the effects of hydration, and hydrophobicity, respectively, on dynamic behavior associated with ssDNA-surface interactions. Since short oligonucleotides are highly constrained due to steric hindrance, while long oligonucleotides have greater conformational freedom, the effects of molecular conformation were explored by studying the interfacial behavior of oligonucleotides as a function of molecular weight.

2.2 Experimental Details

2.2.1 Materials

Methoxy(triethyleneoxy)propyltrimethoxysilane (OEG, 95% pure) and n-octadecyltriethoxysilane (OTES, 95% pure) were obtained from Gelest. n-Butylamine (99.5% pure) was obtained from Sigma-Aldrich. Micro 90 cationic detergent was obtained from International Product Corp. All other chemicals were Optima grade from Fisher Scientific. All chemicals were used as received without further purification or modification. Aqueous solutions were prepared with water purified to 18 MΩ cm⁻¹ using a Millipore Milli-Q system. Alexa488-modified deoxycytidine triphosphate (dCTP, Invitrogen) was used as the single nucleotide probe, and Alexa488-modified poly-cytosine 5-mer, 10-mer, 25-mer, and 50-mer species were used to investigate the effects of ssDNA chain length. DNA solutions were prepared in Milli-Q water to concentrations of 10⁻¹⁰ M to achieve low enough surface densities for single-molecule experiments.
2.2.2 Surface Preparation

Fused silica (FS, Mark optics) wafers were washed in a 2% Micro 90 solution and scrubbed with a Kim-wipe prior to rinsing with copious amounts of Milli-Q water. The wafers were then washed with fresh isopropanol followed by drying with ultrapure nitrogen. Wafers were then placed in a piranha solution (3:1 sulfuric acid:hydrogen peroxide) at approximately 70 °C for 1 h followed again by copious rinsing with Milli-Q water and drying with ultrapure nitrogen. The dry wafers underwent UV-ozone treatment for 1 h. Following the UV-ozone cleaning, FS wafers were either modified with a monolayer of methoxy(triethylenoxy)propyltrimethoxysilane (OEG), n-octadecyltriethoxysilane (OTES), or 3-Glycidoxypropyltrimethoxysilane (GPTMS). To form OEG monolayers via solvent-assisted vapor deposition, a 1:2:20 solution by volume, of n-butylamine:OEG:toluene was placed in the bottom of a 250 ml glass jar. The sample was then placed on the mouth of the jar and the jar was sealed. The sample was left to deposit for 24h at room temperature. The preparation of GPTMS layers followed the same vapor deposition procedure, using a 1:2:20 solution of n-butylamine:GPTMS:toluene for 16-20 hours. n-Octadecyltriethoxysilane monolayers were deposited via liquid deposition for 1 h in a 1:2:20 solution by volume, of n-butylamine:OTES:toluene mixture heated to 70°C in a water bath.

2.2.3 Contact-Angle Measurements

Contact angles (CA) of all surfaces were measured with a custom-built contact-angle goniometer. A 1 µl drop of Milli-Q water was deposited on the surface in seven random locations on three separate samples; the averaged values and standard deviations are reported
below. For unmodified FS samples almost complete wetting of the surface was observed (CA < 5°). The CA of the OEG-modified FS was 18° ± 3° as expected for relatively short methoxy-terminated PEG molecules (MW = 236); longer methoxy-terminated PEG silanes (MW ~ 460-590) have been reported to exhibit somewhat larger contact angles of 36° ± 1°. The CA for GPTMS was 54° ± 3° corresponding to a complete monolayer as measured by ellipsometry as discussed below. The CA of OTES modified FS surfaces was 108° ± 1°, which is characteristic of highly-ordered long-chain self-assembled monolayers.18,19

2.2.4 Ellipsometry Measurements

A variable angle spectroscopic ellipsometer (V-VASE®, J.A. Woolam, Lincoln, NE, USA) was used to measure the thickness of silane thin films in air to assess the monolayer quality. For ellipsometry experiments, 2” intrinsic silicon wafers (WRS Materials) were used as substrates for the thin film deposition described previously; the native oxide on these wafers is expected to be chemically similar to fused silica. An isotropic three interface optical model consisting of air, OEG/OTES, native silicon dioxide layer, and silicon, was used to fit the change in amplitude, $\Psi$, and change in phase, $\Delta$, measured at angles from 60° to 80° at 5° degree intervals, spanning the spectroscopic range from wavelengths of 400nm to 900nm.20 The thickness of the native oxide layer was measured prior to surface modification. This technique gave an OEG layer thickness of 1.62 ± 0.02 nm which is similar to the theoretical length of a fully-extended molecule (~ 1.7 nm) calculated through molecular geometry (using nominal bond lengths and bond angles).21 The layer thickness of GPTMS was 0.9 ± 0.1 nm which is similar to both the fully extended molecular length and previously reported values.22 The layer thickness for the OTES
layer was 1.81 ± 0.02 nm which is similar to typical literature values (~ 1.7 nm) as well as the theoretical length of a fully-extended OTES molecule (~ 2.1 nm).\textsuperscript{23}

2.2.5 \textit{Microscopy Measurements}

Total internal reflection fluorescence microscopy (TIRFM) measurements were performed on a custom-built prism-based microscope consisting of a Nikon TE-2000 microscope with a 60x water emersion objective and a 491 nm DPSS LASER oriented to produce a TIRF field when flow cell and prism are mounted on the microscope. This microscope configuration has been described in detail previously.\textsuperscript{24} The intensity of the illumination was adjusted to permit single fluorophore observation with a 100 ms acquisition time during sequential imaging, while also allowing continuous observation for several minutes without photobleaching. Since it was impossible to accurately measure residence times or diffusive trajectories of objects that resided on the surface for less than one frame (100 ms), all such species were removed from the data pool.

2.2.6 \textit{Photobleaching Rate Determination}

Using the microscope setup described above, photobleaching studies were performed to determine the characteristic photobleaching time under typical imaging conditions. In this study fluorescein was used instead of its more photostable derivative, Alexa-488.\textsuperscript{25} The change in dye was necessary since DNA labeled with Alexa-488 cannot be amine modified due to chemical reactivity between the Alexa dye and amine modifier. Surfaces were initially prepared as described above using the GPTMS modification. Amine-modified ssDNA (16-mer poly-C)
terminally modified with fluorescein, was then covalently attached to the epoxy-modified surface via a primary amine-epoxide ring-opening addition reaction. This was accomplished by immersing the epoxy-modified FS substrate into a pH 8-10, 50nM solution of the amine-modified ssDNA for 20 hours at ambient temperature. These surfaces were then vigorously cleaned using a sequence of rinses with acetone, ethanol, and toluene followed by 10 minute bath in boiling Milli-Q water. Imaging of the surface was then performed under continuous TIRF illumination at laser power similar to standard TIRF experiments. The interval for image acquisition was 4 s and imaging was performed for 600 s. Single molecules were identified on a frame by frame basis, counted, and tabulated versus time. If a molecule disappeared for one or more frames but then reappeared in the same position, that molecule was considered to have undergone a blinking event as opposed to actual photobleaching. The results were then plotted and fit using an exponential decay as expected from a first order kinetics model, (see Supporting information section Figure 2.10). The characteristic photobleaching time (i.e. the inverse photobleaching rate) for fluorescein was determined to be $290 \pm 10$ s. Alexa-488 is more photostable than fluorescein, so this characteristic photobleaching time (290s) represents a lower limit for the photobleaching time constant of Alexa-488 modified ssDNA. Since this time constant is more than an order of magnitude larger than that of the longest residence time mode for Alexa-488 modified ssDNA (~14s), we conclude that the observed residence times were representative of actual desorption events, and not an artifact of photobleaching.
2.2.7 Data Analysis

Diffraction-limited objects were identified by a disk matrix and thresholding algorithm on a frame by frame basis. Center-of-intensity calculations were used to determine object locations in each frame. An object that appeared within 3 pixels (681 nm) in consecutive frames was identified as the same object for purposes of tracking. At least 75,000 molecular trajectories were obtained for each probe molecule on each type of surface. Only objects that were directly observed to both adsorb and desorb were used in the analysis to eliminate uncertainty with respect to the surface residence time. The surface residence time of each object was calculated as the number of frames in which the object was identified multiplied by the exposure time (100 ms) to convert to units of time. Since an object may not reside in the initial and/or final frames for the entire exposure time of that frame, the uncertainty in the residence time was taken to be the exposure time divided by √2.

The cumulative residence time distribution was modeled using a multi-exponential function, where the coefficient of each term represents a population fraction \( f_i \) and the time constant represents the characteristic residence time \( \tau_i \) of that population (Eqn. 2).

\[
p(t) = \sum_i f_i e^{-t/\tau_i} \tag{2.1}
\]

The error for each data point in the distribution represents a 68% confidence interval for a Poisson distribution. Mean residence times were calculated by a weighted average of the
residence times of all observed populations. A more detailed discussion of this analysis was
given previously.\textsuperscript{27}

The squared-displacement was calculated for each step of each trajectory. Experimental
cumulative squared-displacement distributions were created by sorting the squared-
displacement data and ranking each data point \textsuperscript{24}. The error for each data point in the
distribution represents a 68\% confidence interval for a Poisson distribution. The cumulative
distribution was then fitted to a multiple-Gaussian function to extract population fractions ($x_j$)
and their respective diffusion coefficients ($D_j$). A more detailed discussion of this analysis was
given previously (Eqn. 3).\textsuperscript{24,27}

\begin{equation}
C(R^2, \Delta t) = \sum_j x_j e^{-R^2/4D_j t} \quad (2.2)
\end{equation}

2.2.8 Molecular Modeling

ssDNA conformations were explored using Spartan\textsuperscript{®} molecular modeling software. In these
analyses, 5-mer, 10-mer, 25-mer, and 50-mer poly-C chains were created, initially in an
elongated conformation. Each molecule was then folded by rotating the molecule about
chemical bonds in such a way to expose the maximum surface area of the phosphodiester
backbone while attempting to internalize the nucleobases in a “micelle-like” conformation.
Embedded Merck Molecular Force Field (MMFF) energy minimization calculations were run
periodically throughout the folding process to anneal the structure. MMFF calculations account
for bond stretching, angle bending, stretch-bend interactions, out-of-plane bending, torsion
interactions, van der Waals interactions, and electrostatic interactions.\textsuperscript{28} This procedure ignored the inherent stiffness of the ssDNA chain due to electrostatic interactions that lead to a persistence length of \(~2\ \text{nm}\) under the conditions of the experiment.\textsuperscript{29} Therefore, the resulting structures, while indicative of a systematic trend, represent limiting cases that are more compact than the true molecular conformations.

Spartan\textsuperscript{®} molecular modeling software was also used to examine the electrostatic potential map of each of the surface modification ligands. To create an electrostatic potential map, Spartan\textsuperscript{®} combines the electron density and electrostatic potential surfaces. Electron density defines the molecular shape and size, performing a similar function to a space-filling model. The electrostatic potential energy (\(|\Delta U|\)) at a point on the electron density surface is given by the electrostatic potential energy between an imaginary positively charged ion and the molecule. If the ion is attracted to the molecule then the potential is negative. Spartan\textsuperscript{®} calculates the electrostatic potential at selected points along the electron density surface creating a rainbow color coded surface potential map with arbitrary units, creating a model describing the molecule’s size and shape as well as its charge. Using the electrostatic potential map, the polar surface area was measured for each surface-modifying ligand.

2.3 Results and Discussion

Two types of model surface were employed to explore the effects of polarity on ssDNA-surface interactions (Figure 2.2). The OTES represented an effectively non-polar environment, with calculations giving a polar surface area (PSA) of \(0\ \text{Å}^2\)/molecule, and a maximum molecular
surface electrostatic potential of $|\Delta U| \approx 10$ as defined above. This was consistent with the measured value of the water contact angle (108°). The OEG surface represented a hydrophilic case with $|\Delta U| = 210$ and a PSA of 28 Å$^2$/molecule, again consistent with the water contact angle of 16°. The similarities in the molecular lengths of OTES (18.1 ± 0.2 Å) and OEG (16.2 ± 0.2 Å) provided similar opportunities for ssDNA molecules to intercalate within the “soft layer” of both model surfaces.

The cumulative surface residence time distributions for the monomer probe molecule dCTP (C1), and poly-cytosine 5-mer (C5), 10-mer (C10), 25-mer (C25), and 50-mer (C50) on each model surface (OEG and OTES) were calculated as described above from raw molecular

![Figure 2.2 - Chemical structure and electrostatic potential map of model surfaces OTES (top), OEG (bottom).]
trajectories. Representative residence time distributions for C5 on OTES and OEG are shown in Figure 2.3 for temperatures ranging from 282–323K. On these semi-log plots, a straight line would indicate first-order desorption kinetics; the observed deviation from this behavior indicates the presence of multiple populations with unique characteristic residence times. In each instance, a distinct dependence of residence time on temperature was observed. For OTES surfaces (Figure 2.3, top row), the residence time distribution initially shifted “upwards” to longer times with temperature in the range of 283–293K, and then shifted systematically “downwards” to shorter times as temperature was increased further in the range of 293–323K.

Figure 2.3 - Semi-log plots of the cumulative surface residence time distributions of C5, on OTES-modified fused silica (top row), and OEG-modified fused silica (bottom row).
This distinctive non-monotonic trend was observed for the residence time distributions of both C1 and C5 with a peak between 293–303K. This non-monotonic behavior was not observed for the OEG surface. On the OEG surface a slight shift to longer residence times was observed over the entire observed temperature range 283–323K (Figure 2.3, bottom row). The non-monotonic behavior observed on the OTES surface with a peak at 293–303K is a known characteristic of hydrophobic interactions.\textsuperscript{14,16}

The cumulative residence time distributions (terminated at the 99.9 percentile) were fit with a triple-exponential function as described above to characterize each observed population with a characteristic residence time and population fraction (see Table 2.1 and 2.2 in the Supporting Information section for exact values). Interestingly, the population fractions changed only modestly with molecular size. However, significant shifts were observed in the characteristic residence times and these shifts were the dominant factor in changes in the residence time distribution for each probe-surface combination. The characteristic residence times for each mode are shown as a function of molecular length and temperature on OTES (Figure 2.4) and OEG (Figure 2.5).

In all cases, a short-lived fraction, representing the majority of the adsorbing molecules (>70%), exhibited a characteristic residence time in the range 0.15–0.45s. A second population, representing roughly 10–20% of adsorbing molecules had a residence time in the range 0.65–1.90s, and a relatively rare (<4% of all molecules) long-lived population had a residence time of 1.80–15.20s.
Inspection of the characteristic residence times on OTES (Figure 2.4) revealed that most populations of the smaller probe molecules (dCTP and C5) exhibited the non-monotonic temperature-dependence characteristic of hydrophobic interactions. This behavior was not
observed for poly-C longer than C5. The juxtaposition of the non-monotonic temperature dependence of shorter chain species and the linear temperature dependence of longer chain species suggests that shorter chain surface behavior is dominated by hydrophobic interactions while longer chain lengths are not.

Dynamic behavior for varying poly-C lengths on hydrophilic OEG is shown in Figure 2.5, which displays the residence time for each population as a function of temperature and chain length. In contrast to the hydrophobic OTES surface, no non-monotonic temperature-dependence was observed for any probe molecule. In fact, for any given molecular species, only very modest changes with temperature were observed, suggesting that, for all chain lengths and residence modes, the surface behavior on OEG was not dominated by hydrophobic interactions. This is expected since the OEG surface is hydrophilic and should exhibit van der Waals forces as the primary interaction with the probe molecules.

Figure 2.4 and Figure 2.5 also exhibit an interesting (and opposite) dependence of nucleotide-surface interactions on molecular length. OEG surfaces exhibit increasing residence times with increasing ssDNA chain length, while OTES surfaces generally demonstrate the opposite trend (particularly for C10–C50). This dependence on molecular size is visually apparent by scanning across each row of the Figures 4 and 5. A direct comparison of absolute residence time shows that the lower molecular weight cytosine species (C1–C10) reside for longer periods on the OTES surface than the OEG surface, emphasizing the importance of hydrophobic interactions for smaller oligonucleotides. This suggests that short chain species adopt conformations that
allow for the hydrophobic moieties of the amphiphilic ssDNA molecule to interact favorably with the more hydrophobic surface, resulting in longer residence times. However, for the longer C25–C50 species, the opposing trends with molecular weight on hydrophilic and hydrophobic surfaces lead to a situation where residence times on OTES actually become

Figure 2.5 - Characteristic residence time versus temperature for OEG surface. From left to right columns show data for dCTP, C5, C10, C25, C50. Rows from top to bottom represent the long, medium, and short residence time modes respectively.
shorter than on OEG. As discussed further below, we hypothesize that the molecular conformations favored by longer oligonucleotides (e.g. more compact molecular configurations) limit the amount of exposed hydrophobic moieties, reducing the favorable interactions with hydrophobic surfaces, with a concomitant reduction in the surface residence time.

Surface mobility provides complementary information about interactions between probe molecules and surfaces. Previous work by our group suggests that several mechanisms may be involved in surface diffusion. In one mode (“flying”), a molecule undergoing surface diffusion partially or completely detaches from the surface\(^{30, 31}\) permitting the molecule to bypass potential barriers. Another mode (“crawling”) involves local motions without detachment.\(^ {31}\) For a hydrophobic molecule on a hydrophobic surface, the “crawling” mode was found to be essentially activation-less,\(^ {30}\) consistent with a very smooth laterally-homogeneous surface potential and the relatively long-range nature of the hydrophobic interaction, which tends to smooth the lateral variations of the local potential.

Cumulative squared-displacement distributions were calculated for each probe/surface combination as described previously (see Supporting Information section Figure 2.11). These distributions were then terminated at the 99.9 percentile and fit to a double-exponential function as described above to characterize each diffusive mode.\(^ {27}\) The calculated effective diffusion coefficients for each mode are shown in Figure 2.6 versus temperature for each ssDNA molecular length (see Table 2.3 and 2.4 in the Supporting Information section for exact values).
As expected, for all ssDNA-surface combinations, the experimental surface diffusion coefficients were smaller than reported 3D diffusion coefficients in solvent by a factor of 100–1000. The crawling mode diffusion coefficients showed little dependence on temperature or chain length on either hydrophobic or hydrophilic surfaces. The diffusion coefficients for the crawling mode were \(~0.05\ \mu m^2/s\). For small values of the diffusion coefficient, the phenomenon of apparent diffusion becomes increasingly important. Apparent diffusion is the perceived motion of molecules due to the inability to perfectly localize an object spatially and can be estimated by taking the square of the positional uncertainty (\(~60\ nm\) in our system) divided by the frame.

Figure 2.6 - Diffusion coefficients versus temperature for each surface/probe combination. From left to right columns show data for dCTP, C5, C10, C25, C50. Rows from top to bottom represent data for surfaces OTES and OEG.
rate (0.1 s), giving ~0.04 µm²/s in this case. Previous work performed with fibrinogen found that even relatively immobile populations had an apparent diffusivity of similar magnitude. Thus, the measured values of the diffusion coefficient associated with the crawling diffusion mode are near the limit for accurate measurement under the current experimental conditions. Therefore, it would be difficult to draw significant conclusions regarding the temperature dependence of the crawling diffusion mode.

However, the flying mode diffusion coefficients for C1–C10 exhibited a distinctive non-monotonic temperature-dependence peaking at 303K. This non-monotonic behavior is again indicative of dominant hydrophobic interactions between OTES and short-chain poly-C. This non-monotonic behavior was not observed for the C25 and C50 species, whose diffusion coefficients varied only modestly with temperature. Again, a transition was observed at the C10 length from dynamics dominated by hydrophobic interactions to van der Waals interactions. This further supports the hypothesis that longer ssDNA molecules adopt conformations that minimize the amount of exposed hydrophobic surface area available to interact with the surface. In contrast to the behavior on hydrophobic surfaces, the diffusion coefficients of all probes on the OEG surface exhibited a statistically-insignificant dependence on temperature. This lack of temperature-dependence again suggests that, as expected, the surface behavior on OEG was not dominated by hydrophobic interactions for any chain length or diffusive mode.

On OTES surfaces, the mean diffusion coefficient increased systematically with molecular length. In contrast, they decreased with molecular length on OEG surfaces. Since two diffusive
modes were observed for most species, changes in the mean diffusion coefficient can be due to
differences in the diffusion coefficients of the various modes, the fraction of “steps” corresponding
to the different modes, or a combination of both. The fraction of the steps corresponding to
the flying diffusive mode is plotted versus temperature for each probe surface combination in
Figure 2.7. A detailed analysis revealed that both the diffusion coefficients of the flying mode
as well as the mode fractions were sensitive to changes in ssDNA chain length and temperature.
The overall trends in the flying mode fraction shown in Figure 2.7 exactly mirror those in Figure
2.6 for the flying mode diffusion coefficient, suggesting that both the mode fraction and
diffusion coefficient of polynucleotides are affected by surface hydrophobicity. In particular, the

![Figure 2.7 - Fraction of population in flying mode versus temperature for each surface/probe combination. From left to right columns show data for dCTP, C5, C10, C25, C50. Rows from top to bottom represent data for surfaces OTES and OEG](image-url)
increase of the surface mobility on OTES surface with molecular size was due both to the increased diffusivity of the flying mode as well as an increase in the flying mode fraction. Similarly, on OEG surfaces, the diffusivity of the flying mode and the flying mode fraction both decreased with molecular length. Notably, non-monotonic temperature-dependence was again observed for the flying mode fraction of short chains on OTES.

The diffusion coefficient trends observed on OEG and OTES surfaces were consistent with those previously described for the mean surface residence time. For OEG, decreasing mobility and longer surface residence times were both consistent with increasing molecule-surface interactions as a function of molecular length. For OTES, the opposite was true; increasing mobility and shorter surface residence reflected a decreasing molecule-surface interaction with molecular length. In the study by Honciuc et al., it was observed that as surfactant size increased so did the diffusion coefficient on hydrophobic surfaces. Honciuc’s work also demonstrated hydrophobic collapse of the longest chain fatty acid species (BODIPY-(CH₂)ₙ-COOH n ≥ 12) resulted in an anomalously low activation barrier to diffusion. This suggests that the apparent increase in diffusion with increasing ssDNA chain length on OTES is the result of hydrophobic collapse of the long chain DNA into a more compact conformation. While van der Waals interactions increase monotonically with the size of the ssDNA molecule, the hydrophobic collapse of long-chain ssDNA (which sequesters the nucleobases within a hydrophobic core) reduces hydrophobic exposure. As discussed earlier, this would reduce the corrugation of the interaction energy, and therefore the apparent activation energy for diffusion.
These observations are not limited to oligonucleotides containing primarily cytosine residues. It is expected that, for short chain lengths, hydrophobic interactions would influence characteristic residence time and diffusion coefficients for ssDNA of any composition. For example, qualitatively-similar observations were made for poly-adenosine (polyA), where opposing trends in residence time were again observed vs. chain length on OTES and OEG. The cross-over (where residence time on OEG became longer on OEG than on OTES) for polyA occurred for slightly shorter chains than for polyC, consistent with the greater hydrophobicity of adenosine as compared to cytosine. In general, therefore, it is expected that the observed trends would be independent of sequence composition. However, subtle details, e.g. critical chain lengths, are expected to be sequence-dependent.

Interestingly, the temperature trends of short chain species C1–C5 on OTES demonstrated an increase in both residence time as well as flying mode diffusion up to 303 K followed by a decrease in both dynamic parameters. As discussed previously, the extremely slow diffusion associated with the crawling mode made this analysis inconclusive for that mode. One might naively expect that an increase in residence time should always be accompanied by slower surface diffusion. However, recent molecular dynamics studies performed by the Garde group for hydrophobic molecules interacting with surfaces found that as the hydrophobicity of the surface was increased, molecules were bound more strongly, but also exhibited greater lateral mobility. This behavior is presumably due to the orthogonal nature of the energy barriers associated with desorption vs. lateral diffusion. In particular, activation energies associated with desorption \( (E_{des}^u) \) are related to the overall strength of molecular interactions normal to
the surface while energy barriers associated with surface diffusion ($E_{\text{diff}}^a$) are associated with the corrugation of the energy landscape within the plane of the surface. For a surface dominated by short-range forces, such as van der Waals interactions, the energy profile of the surface would be highly corrugated due to short-range lateral variations in surface chemistry, resulting in large barriers to lateral motion (Figure 2.8a). In this scenario, the desorption rate and surface diffusion rate should parallel each other. This complementary behavior is observed for all species on the OEG surface as well as chains C10 and longer on the OTES surface. Conversely, longer range interactions, such as hydrophobic interactions, tend to smooth the

![Schematic energy profiles of surfaces dominated by (a) short range interactions and (b) long range (hydrophobic) interactions. As the strength of hydrophobic interactions increases, the overall depth of the potential increases while also becoming smoother.](image)

Figure 2.8 - Schematic energy profiles of surfaces dominated by (a) short range interactions and (b) long range (hydrophobic) interactions. As the strength of hydrophobic interactions increases, the overall depth of the potential increases while also becoming smoother.
lateral energy profile of a surface for diffusion (Figure 2.8b) leading to the opposite correlation between diffusion and desorption. In particular, an increase in hydrophobic interaction strength at low temperatures leads to both longer residence times and faster diffusion, and the opposite trend as the interaction strength decreases at higher temperatures.

We suggested above that ssDNA may adopt conformations that minimize exposure of the hydrophobic nucleobases to the aqueous solvent. In order to schematically illustrate conformations that ssDNA can potentially adopt as a function of chain length, C5 – C50 molecules were folded in such a way as to minimize the solvent-exposed hydrophobic surface area. The results of this modeling experiment are shown in Figure 2.9. The structure of C5 - C10 is shown in Figure 2.9 and, as expected, the molecules are unable to adopt a structure that completely internalizes the nucleobases when using MMFF energy minimization. Instead, a “linear” structure is adopted for C5, and a “baseball” structure is adopted for C10, leaving a crescent of nucleobases exposed as indicated on the bottom of Figure 2.9. For C25 and C50, on the other hand, it was possible to internalize the nucleobases almost completely, leaving only a few exposed (Figure 2.9, C25).

We emphasize that the structures shown in Figure 2.9 neglect some important structural features of ssDNA. In particular, the persistence length of ssDNA under the conditions of our experiments is expected to be ~2 nm. Using a contour length of ~5.6 Å per nucleotide, this suggests that groups of less than 4 nucleotides could be interpreted as approximately rigid rods. Thus the C5 and C10 species would be relatively rigid and have trouble internalizing the
nucleobases as Figure 2.9 suggests. The longer chains would still be able to internalize the nucleobases though not necessarily in a globular structure suggested by the modeling study. Previous work in the literature has demonstrated that ssDNA can adopt either rod-like or globular conformations on surfaces when deposited out of polar solutions and imaged with atomic force microscopy.\(^{35}\) A molecular dynamics study on ssDNA conformation in water and PBS buffer, by Martinez et al, found that increasing chain length promotes base-base stacking which would also internalize the nucleobases.\(^{36}\) These studies, as well as the molecular modeling described here, support the hypothesis that ssDNA adopts a molecular size limited conformation to hide the nucleobases when interacting with surfaces.

The trend illustrated in Figure 2.9 provides insight into the dynamic interfacial behavior described previously. Naively, one expects that larger molecules will have greater surface attraction, generally consistent with what is observed on hydrophilic surfaces. Indeed,
regardless of whether the molecular conformation is extended, random coil, or micellar, the interaction strength between an oligonucleotide and an OEG surface is destined to increase with molecular length, due to increasing van der Waals contacts. However, on the OTES surface, where hydrophobic interactions are dominant, the hydrophobic overlap between adsorbed oligonucleotide and surface may actually decrease as the molecular size increases due to the enhanced ability of the longer molecule to sequester hydrophobic nucleobase moieties within the molecular interior.

2.4 Conclusions

In contrast with conventional ensemble-averaging techniques, single molecule tracking provides information about individual molecular populations, giving insights into molecular conformation and surface interactions that are otherwise lost. Since certain populations (even rare ones) may be disproportionately important for specific phenomena (e.g. DNA hybridization), this sort of population analysis is critical for the understanding of dynamic behavior of (poly)nucleotides at interfaces. In the experiments performed here, we found that dynamic interfacial properties of ssDNA (residence time and surface diffusion) were inherently dependent on the chain length of the probe nucleotide; sometimes in a non-intuitive way. This may be an important factor when designing genome sequencing or biosensing applications. Since longer oligonucleotides tend to form structures where nucleobases are sequestered internally, surfaces that encourage un-folding of the micelles may promote more efficient hybridization in the near surface region.
Techniques such as sequence-by-synthesis (genome sequencing) use nucleotide triphosphates as probe molecules. In these experiments it is important that molecules have short surface residence times to reduce surface sticking which leads to insertion errors. Thus, hydrophilic surface chemistries, similar to OEG, would appear to be desirable. Other attributes to consider include surface diffusion, which dictates the probe molecule’s ability to explore the surface to target a tethered capture molecule. OEG surfaces have the largest mean diffusion coefficient and thus, the most rapid surface exploration. These two factors suggest that for a sequence-by-synthesis technique using monomeric nucleotides, a hydrophilic non-hydrogen bonding surface similar to OEG is desirable. Due to the unusual temperature dependence of the hydrophobic effect, it is possible to control surface residence and diffusion rates in a system via temperature control. This provides another tool to potentially enhance throughput of microarray techniques. Thus, the information about nucleic acid surface dynamics provided by single molecule tracking gives new insight into the ways in which surface chemistry can be used to improve microarray and biosensor technology using short chain oligonucleotides.
2.5 Reference


(13) Kauzmann, W. *Advances in Protein Chemistry* 1959, 14, 1-63.


(22) Tsukruk, V. V.; Luzinov, I.; Julthongpiput, D. *Langmuir* 1999, 15, (9), 3029-3032.


2.6 Supporting Information

2.6.1 Photobleaching Rate Determination Data

Time-lapse imaging of the surface was performed under continuous TIRF illumination. The time-lapse interval for image acquisition was 4 s and imaging was performed for 600 s. Single molecules were identified on a frame by frame basis, counted, and tabulated versus time. If a molecule disappeared for one or more frames but then reappeared in the same position, that molecule was considered to have undergone a blinking event as opposed to actual photobleaching. The results were then plotted and fit using an exponential decay expected from first order kinetics model. An example figure for fluorescein photobleaching experiments is shown in Figure 2.10.

![Figure 2.10 - Number of molecules versus exposure time with best fit line based on a first order kinetic model.](image-url)
2.6.2  Residence Time Distribution Parameters

Residence time distributions were fit to a tri-exponential function to characterize each observed population with a characteristic residence time and population fraction. The three populations were designated the short, moderate and long residence time modes based on the characteristic residence times. The parameters of the exponential fits for OTES and OEG are given in Table 2.1 and 2.2, respectively.
Table 2.1 - Characteristic residence times for the short, moderate and long residence time modes and their respective population fractions for the OTES surface.

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Table 2.2 - Characteristic residence times for the short, moderate and long residence time modes and their respective population fractions for the OEG surface.

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2.6.3 *Cumulative Squared Displacement Distributions*

Experimental cumulative squared-displacement distributions were created by sorting the squared-displacement data and ranking each data point. The distributions demonstrating the effect of temperature on squared displacement are shown in Figure 2.11 for the C5 probe on both OTES and OEG surfaces, and demonstrate that squared displacement initially increases on the OTES surface up to 30C and then decreases with a continued increase in temperature. A multiple-Gaussian function was fitted to each cumulative distribution to extract population fractions and their respective diffusion coefficients. The best-fit parameters are given in Tables 2.3 and 2.4.
Figure 2.11 - Semi-log plots of the cumulative squared displacement distributions of C5, on OTES modified fused silica (top row), and OEG-modified fused silica (bottom row). Error bars are present in the figure but are generally smaller than the symbols.
Table 2.3 - Diffusion coefficients and respective population fractions for the flying mode of diffusion for each surface/probe combination.

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<th>Population Fraction</th>
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Table 2.4 - Diffusion coefficients and respective population fractions for the crawling mode of diffusion for each surface/probe combination.

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<th>OTES Population Fraction</th>
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Chapter 3: Mechanisms of Surface-Mediated DNA Hybridization

3.1 Introduction

Surface hybridization involves a target single stranded deoxyribonucleic acid (ssDNA) engaging in specific interactions (hybridization) with immobilized probe ssDNA molecules. Various technologies rely on measurements of surface hybridization to sequence DNA, detect DNA polymorphism, DNA mapping, and detect DNA mutation. In principle, hybridization relies on the thermodynamic equilibrium of hybridized and unhybridized DNA. The complex mechanisms behind this equilibrium involve molecular, electrostatic, and conformational interaction phenomena. The complexity of hybridization is exacerbated when considering hybridization between free and immobilized DNA at an interface where probe coverage and surface chemistry must be considered.

While the influence of the vicinal surface is sometimes neglected, it is intuitively reasonable that the desired specific molecular-recognition interactions must compete with non-specific interactions between probe or target molecules and the surface itself. Therefore, the physicochemical properties of the adjacent surface are hypothesized to have a significant impact on the efficiency and kinetics of hybridization and other biomolecular interactions. For example, it was found that the conformation of hairpin DNA was greatly perturbed at both hydrophobic and hydrophilic surfaces relative to solution, and that the hairpin state was more stable on the hydrophobic than on the hydrophilic surface. The rates of folding and unfolding were also significantly slowed at the surface.
While it is clear that hybridization at a surface is not identical to hybridization in solution, a
detailed understanding of surface-mediated hybridization is lacking. Rates of hybridization on
surfaces modified with DNA have been shown to depend on probe length,$^{10}$ design,$^{11}$ and
surface density.$^{7,12-15}$ Classical models that describe the rate of surface hybridization based on a
combination of “specific adsorption” (direct adsorption to immobilized nucleotides), and “non-
specific adsorption” (adsorption to the surface and subsequent surface transport) have been
described.$^{16,17}$ In these models, target molecules may non-specifically adsorb to the surface and
undergo a two dimensional search of the surface for an immobilized complement. Also, the
target molecules may adsorb, diffuse, and desorb without ever hybridizing. Thus the net rate of
surface-mediated hybridization may include both the rate and efficiency associated with the
search process, as well as the fundamental hybridization rate constant. It has been suggested
that the two-dimensional nature of the search may enhance the flux of targets to probe sites,$^{17}$
resulting in more efficient hybridization. In fact, search processes in reduced dimensionality are
generally believed to improve targeting efficiency,$^{18-21}$ and are well-represented in biological
systems.

Ensemble-averaging methods, like cyclic voltammetry,$^{22}$ quartz crystal microbalance$^{10}$ and
surface plasmon resonance spectroscopy,$^{23}$ are commonly used to evaluate the net rate of
hybridization on ssDNA arrays, but are insensitive to the mechanistic details of surface-
mediated hybridization. In fact, generally these techniques do not explicitly distinguish
between specific and non-specific binding. Single-molecule (SM) methods have the potential to
separate and identify competing mechanisms, like those described above, by providing detailed
information about large numbers of molecular trajectories, as opposed to the ensemble average. Total internal reflection fluorescence microscopy (TIRFM) provides the requisite surface sensitivity. When coupled with intermolecular resonance energy transfer (RET), TIRFM represents an analysis tool that can evaluate the entire pathway to hybridization. In particular, the nm-scale range of RET provides a means to unambiguously identify hybridization events. Here it is shown that SM-RET-TIRFM can provide uniquely detailed information about the mechanisms of DNA hybridization in the near-surface environment.

3.2 Experimental Details

3.2.1 Materials

3-Glycidoxypropyltrimethoxysilane (GPTMS, 95% pure) was obtained from Gelest; n-Butylamine (99.5% pure) was obtained from Sigma-Aldrich; Micro 90 cationic detergent was obtained from International Product Corp. All other chemicals were Optima grade from Fisher Scientific. All chemicals were used as received without further purification or modification. Aqueous solutions were prepared with water purified to 18 MΩ·cm using a Millipore Milli-Q UV+ system. The target molecule (introduced into solution) was an oligonucleotide with the sequence 5’-ACAACCAACACACCA-3’ (poly-AC, Invitrogen) with the 5’ end modified with AlexaFluor® 488 dye via C6-Amino linker, (Figure 3.17, top molecule). The probe molecule (attached to the surface) was an oligonucleotide with the sequence 3’-TGTTGGTTGTGTGGT-5’ (poly-TG, Biosearch) labeled with Quasar® 670 dye via 3’ C7-Amino linker on the 3’ end and with an C6-Amino linker on the 5’ end, (Figure 3.17, bottom molecule). The chemical structure of both probe and target oligonucleotides can be found in the supporting information (Figure 3.17). An oligonucleotide
with the sequence 3’-AAAAAAAAAAAAAAA-5’ (poly-A, Invitrogen) with a 5’ AlexaFlour® 488 label attached by a C6-Amino linker was used as a target in control experiments. DNA solutions were prepared in Milli-Q water to concentrations of \(10^{-10}\) M to achieve low enough surface densities for single-molecule experiments. Amine modified Poly-TG DNA solutions were prepared in 1x PBS to concentrations of \(10^{-8}\) M to achieve appropriate surface coverage of covalently attached DNA.

### 3.2.2 Surface Preparation

Fused silica (FS, Mark optics) wafers were washed in a 2% Micro 90 solution and manually scrubbed with a Kim-wipe prior to rinsing with ample amounts of Milli-Q water. The wafers were then rinsed with isopropanol and dried with ultrapure nitrogen. The wafers were subsequently placed in a piranha solution (3:1 sulfuric acid:hydrogen peroxide) at approximately 70 °C for 1 h followed by copious washing with Milli-Q water and drying with ultrapure nitrogen. The dry wafers were then treated with UV-ozone for 1 hour. After the UV-ozone cleaning, 3-glycidoxypropyltrimethoxysilane (GPTMS) was used to deposit a monolayer on the FS wafers.

To form GPTMS monolayers via vacuum-assisted vapor deposition, a 1:2:20 solution by volume, of n-butylamine:GPTMS:toluene was placed in the bottom of a desiccator. The FS sample was placed in a custom holding tray in a desiccator, which was then sealed and evacuated. The deposition occurred over 16-20 hours at room temperature. The deposition time was selected
based on systematic characterization using contact angle and ellipsometry (Supporting Information Figure 3.18).

To modify surfaces with immobilized ssDNA, GPTMS surfaces were prepared as described in the Experimental Details. Surface quality was verified with ellipsometry and contact angle before depositing the amine modified poly-TG DNA. High quality GPTMS samples were incubated for 24 hours in a 1x PBS solution of 100nM poly-TG DNA with the pH adjusted to 8-10. At the end of the deposition samples were rinsed consecutively with toluene, acetone, isopropanol, and Milli-Q water. Following the rinsing sample where placed in a hot water bath ~353K for 15 minutes to ensure only covalently attached ssDNA remained on the surface.

3.2.3 Contact-Angle Measurements

Contact angles (CA) of all surfaces were measured with a custom-built contact-angle goniometer. A 1 µl drop of Milli-Q water was deposited on the surface in seven random locations on three separate samples; the averaged values and standard deviations are reported. For unmodified FS samples, almost complete wetting of the surface was observed (CA < 5°). The CA for GPTMS was 54° ± 3° corresponding to a complete monolayer, measured by ellipsometry as discussed below.\textsuperscript{24}

3.2.4 Ellipsometry Measurements
Variable angle spectroscopic ellipsometry (V-VASE®, J.A. Woollam, Lincoln, NE, USA) was used to measure the thickness of silane thin films in air to assess the monolayer quality. For ellipsometry experiments, 2” intrinsic silicon wafers (WRS Materials) were used as substrates for the thin film deposition described previously; the native oxide on these wafers is expected to be chemically similar to fused silica. An isotropic three interface optical model consisting of air, GPTMS, native silicon dioxide, and silicon, was used to fit the change in amplitude ($\Psi$), and change in phase ($\Delta$) of the polarized light measured at angles from 60° to 80° at 5° degree intervals, spanning the spectroscopic range from wavelengths of 400nm to 900nm. The layer thickness of GPTMS was 0.9 ± 0.1 nm which is similar to both the fully extended molecular length and previously reported values.

3.2.5 Total Internal Reflection Fluorescence Microscopy Measurements

A custom-built prism-based microscope comprised of a Nikon TE-2000 microscope with a 60x water immersion objective was used to preform total internal reflection fluorescence microscopy (TIRFM) measurements. A 491 nm DPSS LASER was oriented to produce a TIRF field when both flow cell and prism were mounted on the microscope, the configuration of which was described in detail previously. The intensity of the illumination was adjusted to permit single fluorophore observation with a 100 ms acquisition time during sequential imaging, while also allowing continuous observation for several minutes without photobleaching. Movies were 1200 frames in length (120s), six movies were taken per sample, and experiments were run in triplicate. An Optosplit II image splitter (Cairn Research) was used for dual channel imaging. This device split the image via a 610 nm dichroic mirror; the dual images were then
filtered by band pass filters optimized for the AlexaFluor®-488 and Quasar®-670 dyes. The donor channel (Alexa-488) used a bandpass filter centered at 529 nm with a 90% transmission width of 28 nm and the acceptor channel used a bandpass filter centered at 630 nm with a 90% transmission width of 50 nm. The two output channels were captured using an EMCCD camera cooled to −70 °C (Photometrics). The two channels were manually aligned, via alignment grid, to within 1-2 pixels. Images were further aligned during post-processing by convolving the two channels as described previously by Kastantin et al.²⁷ Both poly-AC complementary target ssDNA and poly-A control ssDNA experiments were observed at temperatures 293K, 303K, and 313K using a temperature controlled stage. All objects that resided on the surface for less than one frame (100 ms) were removed from the data pool, as it was impossible to accurately measure state times of objects that resided on the surface for less than one frame.

3.2.6 Surface Coverage of Immobilized ssDNA

TIRFM was used to measure the surface density of immobilized probe ssDNA. For surface density experiments, serial dilutions of amine modified poly-TG DNA were prepared ranging from 100 nM – 100 pM. GPTMS surfaces were prepared and incubated in each dilution. After rinsing and hot water bath the samples were examined with TIRFM, the immobilized ssDNA was directly excited with a 643 nm wavelength laser. Images were taken with 1s acquisition times, individual ssDNA molecules were identified as diffraction limited objects. The number of immobilized ssDNA molecules was counted, and the image intensity was integrated to get the total intensity of each image. Over twenty locations were examined on each sample and experiments were run in triplicate for each dilution. Using the collected data a calibration plot
of intensity *versus* surface density was prepared. At greater surface densities of ssDNA it became impossible to identify individual molecules. Therefore, assuming that linearity was retained, the calibration plot was extrapolated to higher total intensities to extract an estimated surface density of ssDNA. For samples prepared using the 100nM ssDNA concentration the grafting density of ssDNA was estimated to be $\sim 1.5 \times 10^9$ DNA/cm$^2$ which equates to an average separation of $\sim 300$ nm between immobilized ssDNA strands.

### 3.2.7 Resonance Energy Transfer

Spatial information in fluorescence imaging was acquired *via* resonance energy transfer (RET). This technique involves the non-radiative energy transfer from a donor fluorophore (Alexa-488) to an acceptor fluorophore (Quasar-670) that occurs when the two fluorophores are within 1-10nm,$^{28}$ where the specific distance-dependence is characteristic of the specific RET-pair. For RET to occur, the emission spectrum of the donor fluorophore (Alexa-488) must have significant overlap with the excitation spectrum of the acceptor fluorophore (Quasar-670). The spectral overlap integral and relative orientation of the fluorophores can be combined with the optical properties of the medium to calculate the Förster radius ($R_0$).$^{28}$ This radius is the distance where the non-radiative energy transfer between donor and acceptor fluorophores is 50%.$^{29}$ The Förster radius for the Alexa-488/Quasar-670 RET pair is approximately 5.4 nm, the exact value is dependent on the molecular orientation ($\kappa^2$) and medium conditions ($n$).$^{30}$ The Förster radius coupled with the experimentally observed fluorescence intensity of the donor ($F_D$) and acceptor ($F_A$) can be used to calculate the spatial separation of the RET pair.
\[ r = R_0 \left( \frac{F_{A,r=0}}{F_{D,r=\infty}} \right)^{1/6} \left( \frac{F_D}{F_A} \right)^{1/6} \]  \hspace{1cm} (1)

The ratio of acceptor fluorescence at zero separation and donor fluorescence at infinite separation is expected to be on the order of unity. Due to the uncertainty in this assumption, as well as those described above, it is challenging to calculate an exact value for \( r \) during RET experiments. Therefore, the relative distance between donor and acceptor fluorophore (\( d \)) was used to characterize DNA hybridization events.\(^{27}\)

\[ d = \left( \frac{F_D}{F_A} \right)^{1/6} \]  \hspace{1cm} (2)

The relative distance \( d \) is directly related to the RET efficiency through the expression:

\[ E = \frac{1}{1 + d^6} \]  \hspace{1cm} (3)

While the RET signal can equally well-described using either \( d \) or \( E \), we have chosen to express our data in terms of \( d \). As a quantity that is proportional to the physical donor-acceptor separation, \( d \) provides a degree of physical intuition not given by \( E \). Moreover, in graphical representations of data, the use of \( d \) provides enhanced sensitivity to extreme values (low and high) of RET, which would be difficult to visualize if represented in terms of \( E \).

3.2.8  Donor Bleeding and Direct Acceptor Excitation

A common artifact of two-channel fluorescent imaging, known as bleeding, involves a small amount of donor emission that may appear in the acceptor channel. This can be reduced to extremely low levels by appropriate selection of dichroic mirror and band pass filters. To assess
the effects of bleeding on the experiments, a control experiment was conducted using donor-labeled target ssDNA with unlabeled complementary probe ssDNA covalently attached to a GPTMS surface. These experiments exhibited strong fluorescence in the donor channel with signals below the noise threshold in the acceptor channel. Therefore, all fluorescence in the acceptor channel can be attributed to RET or to direct excitation of the acceptor fluorophore.

Direct acceptor excitation occurs when the excitation laser (meant to excite the donor to initiate RET) excites the acceptor fluorophore directly with low efficiency, causing the molecule to fluoresce in the absence of RET. To assess the effects of direct excitation a control experiment was performed using unlabeled target ssDNA and acceptor-labeled immobilized probe ssDNA. Using the filters described previously, no fluorescence above background levels was observed in either the acceptor or the donor channel. These control experiments indicate that the system and experimental design limited direct excitation and bleed-through to levels below the noise threshold.

3.2.9 Non-Specific Resonance Energy Transfer

While RET between donor-labeled target and acceptor-labeled probe was intended to indicate a hybridization event, it was expected that some non-specific target-probe interactions would also occur. To characterize these non-specific interactions, a control experiment was performed using the donor-labeled poly-A target (which was not complementary to the acceptor-labeled immobilized probes) in place of the complementary target poly-AC strands. As
described below, the analysis of these data was used to characterize the spectral and dynamic signatures of false hybridization populations.

3.2.10 Data Analysis

Diffraction-limited objects were identified in each channel on a frame-by-frame basis using a disk matrix and thresholding algorithm. For each frame, both channels where convolved to identify objects in the same position. An object’s location was identified using center-of-intensity calculations. The total fluorescence intensity of each object was determined by integrating the intensities of all pixels associated with the object, and subtracting the local median background intensity. An object that appeared within 3 pixels (681 nm) in consecutive frames was identified as the same object for purposes of tracking. Figure 3.1 illustrates the process used to identify RET events that indicated hybridization. Objects simultaneously identified within 2 pixels (455 nm) in both donor and acceptor channels was identified as a RET event, (Figure 3.1 Top). The position of a RET event was identified using the channel with greatest signal to noise ratio. Fluoroscence intensity was monitored in both channels for each molecular trajectory (Figure 3.1, Middle). These intensities were used to calculate the relative distance between fluorophores, $d$, using eq.1 on a frame-by-frame basis. As described below, trajectories were segmented into “searching” and “hybridized” time intervals for the statistical analysis of searching, hybridization, and melting kinetics.
In cases of extremely strong or extremely weak energy transfer, the intensity of a given object was high in one channel but below the noise threshold in the opposing channel. In these cases, the position of the identified object from the strong channel was applied to the weak channel to determine the effective intensity of the object in the weak channel. These objects were tracked as described earlier using a 3 pixel (681 nm) identification radius. At least 100,000 molecular trajectories were obtained for the target and control molecule on the surface. Only objects that were directly observed to both adsorb and desorb were used in the analysis to reduce uncertainty with respect to the surface state times. A given surface state time of each object (e.g. searching or hybridized) was calculated as the number of frames in which the object was identified in that state multiplied by the acquisition time (100 ms). Since an object may not reside in the initial and/or final frames for the entire exposure time of that frame, the uncertainty in a given state time interval was taken to be the exposure time divided by $\sqrt{2}$.

**Figure 3.1** - (Top) Raw data images, (Middle) donor and acceptor emission intensity, (Bottom) physical interpretation of observations. In all parts of the figure, donor fluorophores are represented in green and acceptor fluorophores in red.
Cumulative state time distributions were created by accumulating trajectories and examining the time spent hybridized, or searching for a complementary strand within each trajectory. Time steps in the hybridized state were identified by a high RET efficiency (i.e. low $d$). The number of time steps taken before hybridization was multiplied by the acquisition time (100 ms) to determine the searching time interval. State-time distributions were created by accumulating these time intervals for all observed hybridization and searching events. Similarly, this analysis can identify searching intervals by a low RET signal (high $d$), and used to create the cumulative search time distribution. The error for each data point in the distribution represents a 68% confidence interval for a Poisson distribution.

The cumulative state time distributions were modeled using a multi-exponential function, where the coefficient of each term represented a population fraction ($f_i$) and the time constant represented the characteristic state time ($\tau_i$) of that population.

$$p(t) = \sum_i f_i e^{-t/\tau_i} \quad (3.4)$$

The number of exponentials used in the fit was determined via the maximum entropy method. The maximum entropy method is a model-independent method that utilizes Laplace transforms to invert a distribution into a spectrum of characteristic rates. Discrete peaks were identified in this rate-spectrum, and enumerated to determine the appropriate number of populations to use when applying Equation 3.4 in a given analysis. This process has been
employed to identify modes of DNA surface dynamics, and conformations in surface residence time distributions. Mean state times were calculated by a weighted average of the characteristic state times ($\tau_i$, determined from the fit to Equation 3.4) of all observed populations. A more detailed discussion of this analysis was given previously.

Molecular trajectories and portions of trajectories were partitioned, based on the history of each molecule, to acquire a picture of the pathways of surface-mediated DNA hybridization. As one example, each trajectory was scanned for the presence of at least one hybridization event, identified by a transition from a frame with high relative donor-acceptor distance ($d$) to a low $d$ in a subsequent frame (Figure 3.1). The specific threshold used for this analysis is described below in greater detail. From this, the fraction of molecules that hybridized during the course of their trajectories on the surface was determined. Further analysis was performed upon this hybridizing fraction. For example, the set of trajectories that hybridized at some point during their trajectories was sub-partitioned into trajectories that hybridized immediately upon adsorption (indicated by a high RET efficiency in the first observed frame) and trajectories where hybridization occurred after non-specific adsorption. Various other populations were isolated by monitoring trajectories and changes in the relative distance between donor and acceptor fluorophores, to develop a picture of the mechanistic pathways associated with DNA hybridization in the near-surface region.

Each molecular trajectory was split into periods spent hybridized, or searching for a complementary strand. These trajectory fractions were counted to determine the total number
of hybridized-state events and searching-state events. The hybridized-state and searching-state events were further categorized based on the transition to a subsequent state or from a preceding state. For example, a hybridized molecule could melt either into solution (simultaneous melting and desorption) or, to the searching state (unhybridized surface-bound molecules). Using melting into solution as an example of categorization by subsequent state, the number of molecules that melted into solution was divided by the total number of hybridized-state events to acquire the fraction of melting events that involved melting into solution. As an example of categorization by previous state, the number of molecules that melted into solution was divided by the total number of molecules that desorbed; providing the fraction of desorbed molecules that came from the melted state. Using this approach, the fractions of molecules in each state that transitioned to or from either of the other states were determined. This is clearly demonstrated in the results section below.

The fractional pathway analysis was adjusted to account for the finite time resolution of the experiments. Without this adjustment, certain pathways (i.e. those originating in adsorption or ending in desorption) would be under-counted. For example, below the time resolution it is impossible to distinguish between a molecule that hybridized directly upon adsorption from solution and a molecule that adsorbed non-specifically but hybridized quickly so that the search time could not be resolved. To compensate for this limitation, an adjustment was performed using cumulative distributions of time intervals associated with the relevant state for these transitions. Specifically, a given state-time distribution was extrapolated backward to a state time of 0 s using parameters determined in the fitting process described above. The fraction of
molecules missed was then determined by integration from 0-0.1s (the approximate time resolution of the experiment). This fraction was then used to adjust the fraction of molecules that underwent a transition, *i.e.* melting into solution.

### 3.3 Results and Discussion

Spatial information in fluorescence imaging was acquired via resonance energy transfer (RET). This technique involves the non-radiative energy transfer from a donor fluorophore to an acceptor fluorophore that occurs when the two fluorophores are within 1-10nm.\(^{28}\) In our experiments, amine modified ssDNA labeled with the acceptor fluorophore was attached to an epoxide modified silica surface. Aqueous solutions (1x PBS, pH = 7.4) of the donor labeled complementary strand were prepared. Dual-channel image sequences were acquired of the RET labeled complementary strands at the interface between ssDNA modified silica and aqueous solution. Over 600,000 molecular trajectories were determined for the complementary strands; however, molecules that resided on the surface for 100ms (1 frame) or less were removed from the analysis. Figure 3.1 illustrates the process used to identify RET events that indicated hybridization. The presence of objects simultaneously identified within 2 pixels (455 nm) in both donor and acceptor channels was identified as a RET event, (Figure 3.1 Top). The position of a RET event was identified using the channel with greatest signal to noise ratio. Fluorescence intensity was monitored in both channels for each molecular trajectory (Figure 3.1, Middle). We identified whether a molecule was “searching” or “hybridized” by the relative distance between donor and acceptor fluorophores, \(d = (F_D/F_A)^{1/6}\), where \(F_A\) and \(F_D\) are the fluorescence intensities of acceptor and donor species, respectively. The relative distance
between donor and acceptor fluorophores relates to RET efficiency \( (E) \) by the following expression, \( E = \frac{1}{1+d^6} \). A detailed derivation and discussion of these parameters is described in the Experimental Details.

3.3.1 Identification of searching and hybridized states

It was hypothesized that hybridized oligonucleotides could be distinguished from searching oligonucleotides using an appropriate criterion (a “threshold”) based on RET efficiency \( (E) \) or, equivalently, a sufficiently low value of the relative donor-acceptor distance, \( d \). In order to verify the presence of these distinct states, and to establish appropriate criteria for distinguishing states, statistical distributions of all observed \( d \) values were created for all trajectories in a given experiment (Figure 3.2). In Figure 3.2, three distinct populations were observed, zero-RET, high-RET, and complete-RET. The extreme \( d \)-values of zero-RET and complete-RET observations could not be determined quantitatively. Zero-RET occurred in the

![Figure 3.2 - Distribution of the relative donor-acceptor distance for all RET observations in experiments involving complementary DNA at 293K.](image)
limit where the distance between donor and acceptor fluorophores was so large that an extremely large $d$ value (low RET efficiency) was observed. This population is represented by the large bin for $d > 1.8$ (equivalent RET efficiency, $E < 0.03$) in Figure 3.2. Physically, this relatively small population corresponded to searching (unhybridized) donor-labeled target molecules appearing in only the donor channel. Similarly, a small population for complete-RET was resolved for $d < 0.5$ ($E > 0.98$), shown as a single bin in Figure 3.2, corresponding to the population that appeared only in the acceptor channel. The final population was indicated by the prominent peak centered at $d = 0.9$ ($E = 0.65$) with the range $0.5 < d < 1.8$. As discussed below, this central peak is consistent with expectations for the nominal hybridized state.

To calculate the theoretically-expected RET efficiency for the hybridized state, one must consider the structural details of the labeling, including the presence of flexible linkers used to tether the fluorophores to the DNA. A 3-dimensional self-avoiding random walk was used to approximate the length, and the variance of the length, of each fluorophore/linker combination. Using these lengths, along with the diameter of the dsDNA to which the linkers were attached, the approximate donor-acceptor distance was calculated to be 4.4 nm, which equates to $d = 0.81$ ($E = 0.78$) with a variance $\sigma^2 = 0.12$. Notably, these values are consistent with the position and width of the central peak in Figure 3.2, suggesting that this central peak represents the population of nominally-hybridized molecules. A similar distribution of $d$ values was determined for experiments with non-complementary poly-A control target molecules; this distribution is shown in supporting information Figure 3.12. The poly-A distribution exhibited a
small number of RET events for values of $d$ in the range 1.5-1.6 ($E = 0.08 - 0.06$), presumably due to incidental RET interactions.

The choice of the hybridization threshold criterion involved a compromise between two opposing considerations. By selecting a lower hybridization threshold, the risk of capturing incidental RET and RET due to noise was minimized. However, an artificially low value for the hybridization threshold would lead to identification of false searching states, where the duplex may actually be “peeling” briefly but not truly dissociating. To address this issue, the data set, using the complementary probe ssDNA, was mined for events where molecules underwent an apparent “fluctuation,” involving hybridization, dehybridization, and re-hybridization in successive frames. Rehybridization was identified when a molecule hybridized in $\leq 200$ms following dehybridization. A sensitivity analysis was performed where the hybridization threshold was systematically increased from 0 to 100 (Figure 3.13). As expected, the fluctuations reached a maximum at $d = 0.9$ ($E = 0.65$), matching the maximum of the $d$ distribution peak. In the range of $d = 1.6 - 2.0$ ($E = 0.06 - 0.02$) fluctuations varied by less than 10% from the number of fluctuations at $d \geq 10$ ($E \geq 10^{-6}$). Based on these observations, a criterion was defined where the threshold between the searching and hybridized states was defined as $d = 1.6$. While this choice was somewhat arbitrary, and a different threshold would result only in small changes to some numerical values reported below (e.g. the number of hybridization events), the main conclusions of this paper were found to be robust with respect to small variations in the hybridization threshold criterion.
3.3.2 Phenomenology and Statistics of Hybridization and Melting

The criterion described above allowed us to identify molecular association events on the basis of RET efficiency; this was further used to identify dynamic transitions between searching and hybridized states from RET trajectories. Figure 3.3 shows a number of representative trajectories, where the $d = 1.6$ criterion is indicated by a solid red line. In each trajectory, a

![Figure 3.3 - Representative molecular trajectories. The red lines denote the criterion defined as the threshold for hybridization, i.e. $d>1.6$ indicates a freely-diffusing (searching) target molecule while $d<1.6$ indicates close proximity between target and immobilized probe.](image-url)
given donor-labeled target molecule was followed over time as it initially adsorbed and then proceeded to diffuse on the surface, sometimes hybridizing and/or melting (possibly multiple times), and eventually desorbing from the surface. Time intervals where $d < 1.6$ represented periods of time where the target was in close-proximity (possibly hybridized) to an acceptor-labeled immobilized probe molecule. As indicated in Figure 3.3, molecular trajectories were highly varied. In some cases the initial observation was for $d > 1.6$, indicating adsorption into an unhybridized (searching) state. However, for other trajectories, RET was observed immediately upon adsorption ($d < 1.6$), suggesting adsorption in very close proximity to an acceptor-labeled probe. Trajectories subsequently exhibited diverse behavior, including transitions between searching and hybridized states (the $d$-value crosses the red line), and desorption back into solution (complete loss of a given object in both spectral channels).

Approximately 600,000 individual trajectories were determined for complementary donor-labeled target molecules and for control target molecules consisting of donor-labeled poly-A. For target molecules with a complementary sequence to the probe, ~40% of trajectories (~250,000) exhibited at least one significant RET event with $d < 1.6$, while RET was observed for only ~6% of trajectories for the control poly-A oligonucleotide target. This suggested that the majority of RET events for the complementary target did in fact represent hybridization. The time-averaged hybridized fraction was calculated by integrating the overall distribution of RET efficiency and determined to be 21%. As expected, this hybridized fraction observed on the surface was significantly smaller than what would be observed in solution for molecules of similar length and composition, (<80% hybridized). The significantly smaller hybridization
fraction at the solid-liquid interface is consistent with results of DNA hairpin surface behavior, where DNA-surface interactions tended to destabilize the DNA duplex. The 6% of RET events associated with the control poly-A target had a mean duration of only 0.1s (the acquisition time of a single “frame”), consistent with the hypothesis that these RET events represented incidental proximity during random target diffusion. As discussed in detail below, the temporal statistics of RET events for complementary target molecules were significantly different, and included long-lived associations.

The molecular trajectories that exhibited apparent hybridization (~40% of all observed species) were segmented into “searching” and “hybridized” states on the basis of d as described above; each trajectory was also assumed to begin and end with an “in-solution” state. The transition from the “in-solution” state was identified by a molecule adsorbing to the surface, which was indicated by a molecule’s initial appearance in the field of view. Desorption into the “in-solution” state was associated with the disappearance of a molecule from the field of view.

Transitions between the three states were enumerated and normalized by the destination or source state for both the complementary target and control sequence to determine the relative likelihoods (fractional pathways) of these various transitions. A more detailed discussion can be found in the Experimental Details. This process was performed for several temperatures over the range 293 – 313 K (Supporting Information Figure 3.14). A paired student-t analysis revealed that all fractions were statistically identical as a function of temperature, suggesting that the fractional pathways to hybridization were insensitive to temperature over the range of
293-313K. Therefore, the fraction associated with each transition was averaged across the temperature range and shown as percentages in Figure 3.4. As discussed below, the temporal behavior associated with transitions between states did exhibit a systematic trend with temperature.

Analysis of the pathways to hybridization in Figure 3.4 shows that 31% of complementary target molecules adsorbing to the DNA-modified surface hybridized immediately. A comparison with the control strand suggests that ~4% of these events were actually due to incidental RET. The remaining 69% of complementary target molecules adsorbed non-specifically on the surface and did not initially engage in RET with an immobilized probe molecule. Of these non-specifically adsorbed molecules, only 7% executed a successful search and hybridized (~1% of those species were incidental RET events as indicated by the control experiments), with the

![Figure 3.4 - Fractional pathway for DNA surface hybridization. (left) Poly-AC complementary strand (right) poly-A control. An arrow pointing to a state (searching, hybridized, in solution) represents the percentage of molecules entering that state. An arrow leaving a state represents the percentage of molecules leaving that state.](image)
remaining 93%, desorbing prior to hybridization. Similarly, the melting of surface-bound DNA favored melting into solution (77%) over melting to the surface (23%). Molecules that melted onto the surface could then either rehybridize or, in the case of an unsuccessful search, desorb into solution and perform a 3-dimensional diffusive trajectory (potentially including a large lateral displacement) prior to re-adsorption and a renewed search for a complementary strand.

The search and exploration process described above suggests that hybridization efficiency depends on a complex mixture of the adsorption rate from solution, the surface mobility of target molecules, the surface residence time of adsorbed target molecules, the effectiveness of collisions (*i.e.* the fraction of collisions that lead to hybridization), and the rate of dsDNA melting in the presence of a given surface chemistry. It is interesting to consider these phenomena in the context of previous observations of DNA hybridization at surfaces. For example, Chan *et al.* found that an increased surface density of immobilized probe molecules on the surface could actually decrease the efficiency of hybridization. This was interpreted as being due to reduced hybridization efficiency caused by overcrowding; our observations suggest that other factors could be at work, including the influence of DNA surface density on the mobility and desorption of non-specifically adsorbed target. It was previously reported that ssDNA surface dynamics were strongly influenced by the physicochemical properties of a surface, including hydrophobicity. In particular, ssDNA strands exhibited shorter residence times on hydrophobic surfaces, potentially influencing the time intervals associated with 2-dimensional searches for a complementary strand. Hydrophobic surfaces were also shown to increase the rate of hybridization, and to slow the rate of melting, for DNA hairpins. These observations suggest that an optimal surface chemistry would enhance the hybridization
efficiency of non-specifically adsorbed molecules, potentially by improving the search process, while simultaneously stabilizing the hybridized state. Follow-on work will further explore this possibility by carefully controlling (and varying) the chemistry of the surface “matrix” surrounding the immobilized ssDNA. The results of single-molecule TIRF-RET experiments on these surfaces will provide insight into the effects of surface chemistry (e.g. surface hydrophobicity) on the mechanisms of hybridization at the solid-liquid interface.

3.3.3 *Time Intervals of Searching and Hybridized States – Search Times and Melting Kinetics*

In addition to revealing the different mechanisms associated with surface hybridization and melting, a detailed analysis of molecular trajectories also provided kinetic information about search and melting processes. In particular, each state time for a given molecule was determined by counting the number of frames the molecule remained either hybridized or searching before transitioning to the opposing state or into solution. Trajectories for each transition were accumulated to prepare cumulative state time distributions. Each distribution was fit with 1-3 exponentials, the number of exponentials was selected via the maximum entropy method as described in the Experimental Details. From these fits the characteristic state time and associated population fraction were extracted. Figure 3.5 shows cumulative probability distributions for time intervals associated with specific transitions. The data presented in Figure 3.5a, for example, provide statistical information about the time intervals associated with the searching state. Figure 3.5a comprises all searching events including those molecules that had melted and initiated a new search of the surface. In particular, the graph
shows, on a semi-logarithmic scale, the fraction of molecules that search the surface for time \( t \) or longer. Similarly, Figure 3.5b and 3.5c show the fraction of molecules remaining in the hybridized state for time \( t \) or longer, before apparently melting into solution (Fig. 5b) or onto the surface (5c), respectively. In Figure 3.5, the solid circles represent data from experiments using complementary target ssDNA (at 293K), and the red lines indicate fits to one or more

**Figure 3.5 - Cumulative state time distributions for complementary target molecules (●) and fits as described in the text (−).**

(A) transition from searching state to hybridized, (B) desorption from hybridized state, (C) Transition from hybridized to searching state.
exponentials as described above and in more detail in the Experimental Details. The parameters resulting from these fits are given in detail in the Supporting Information (Table 3.1).

On the semi-logarithmic plots in Figure 3.5, a straight line would indicate first-order kinetics; any observed deviation from this behavior therefore suggests the presence of multiple populations with distinct characteristic state times. The search time distribution (Figure 3.5a) shows a linear dependence on the semi-logarithmic axes, and the majority of molecules searched for only fractions of a second before hybridizing; 99.9% of all observed molecules that hybridized did so in < 1s. This suggested that the local 2-dimensional search for a proximal complementary strand was a rapid process. Using the maximum entropy method outlined in the Experimental Details, a single exponential fit was found to be appropriate, and the best fit characteristic search time was determined to be 0.111 ± 0.001 s.

Once a molecule had hybridized, the transition to either the searching or in-solution state was slow by comparison to the search process. This is indicated by the relatively high probability of long state times in the cumulative hybridized state time distributions (Figure 3.5b, c). These distributions were clearly nonlinear on the semi-logarithmic axes, and exhibited long tails extending to time intervals of >10 s. The distribution describing melting into solution (Fig. 5b) required a sum of three exponential functions (three melting modes) for a satisfactory fit, while the distribution describing melting onto the surface (Fig. 5c) was fit with two melting modes. For these two melting processes, the two primary modes of melting had similar time scales:
~15s for slow mode and ~1.4s for the fast mode suggesting that the kinetics of DNA melting was independent of destination. The distribution describing melting into solution also exhibited a third transient mode with a very short characteristic state time of ~0.1s. As shown in Figure 3.6, a similar state-time analysis using the poly-A control target revealed a single characteristic time of ~0.1s, suggesting that this short-lived mode represented the dissociation of a transient non-specific interaction between ssDNA strands as opposed to actual melting of hybridized strands. Other state time distributions for the control target are shown in the supporting information, Figure 3.15.

All state time distributions were fit to multiple exponentials, as described in the Experimental Details, to determine the characteristic state time and fraction of each population. The most relevant parameter values from the fits are given in Figure 3.7 with their associated physical interpretation (see Table 3.1 in the supporting information for further detail.). As mentioned previously, the search-time distribution (i.e. the search time interval distribution) was well-described by a single exponential with a characteristic time of only 0.111 ± 0.001 s. This rapid

![Figure 3.6 - Cumulative hybridized state time distributions for complementary poly-AC target (●) and the poly-A control (Δ).](image)
search time suggested that molecules that adsorbed to the surface performed a brief 2-dimensional search for a complement strand. If a complementary strand was not encountered quickly, the molecule desorbed and executed a 3-dimensional trajectory before repeating the process. Using a typical mean diffusion coefficient determined for a model hydrophilic surface \( (0.18 \mu m^2/s) \), it was estimated that a characteristic search distance to hybridization was approximately 0.08 um, i.e. a target molecule must adsorb within 80 nm of an immobilized probe molecule to have a high probability of hybridization. If hybridization did not occur, the target molecule desorbed from the surface with a characteristic time scale of \( \sim 2 \) s.

The only state time distribution found to have significant temperature dependence was the distribution associated with the melting of the hybridized state. Figure 3.8 shows cumulative hybridized state time distributions as a function of temperature. Consistent with general expectations for DNA melting, the probability of long hybridized time intervals decreased systematically with increasing temperature. This was expected, because as the temperature
approached the solution melting temperature (~323 K, salt adjusted), hybridization events were expected to be less stable and exhibit shorter state times. Again, characteristic state times and population fractions were extracted through (multi)exponential fits (see supporting information Table 3.1 for details). Interestingly, while the state-time corresponding to the shorter-lived hybridized state was insensitive to temperature (remaining at ~1.5 s), the characteristic state time of the longer-lived hybridized state decreased systematically from ~15 s to ~8.3 s as the temperature increased from 293K to 313K. This systematic decrease was consistent with expectations close to the bulk melting temperature, providing the first hint that the longer-lived “hybridized” population may be an accurate representation of the nominal DNA duplex state, while the anomalous behavior of the shorter-lived state suggests that these transient associations may not represent true (or complete) hybridization. An Arrhenius analysis of the longer-lived hybridized mode revealed that melting to the surface exhibited an apparent

Figure 3.8 - Semi-logarithmic plots of the cumulative hybridized state time distributions for Poly-AC targets at 293 K (●), 313 K (■), and 323 K (▲)
activation energy of $E_a = 23 \text{ kJ/mol}$ and melting into solution an apparent activation energy of $E_a = 22 \text{ kJ/mol}$. The Arrhenius plots used in these calculations are found in the supplementary information (Figure 3.16). These values are somewhat smaller, but on the same order of magnitude, as those reported in the literature for similar length strands in solution, $\sim 40 \text{ kJ/mol}$.$^{39, 40}$ These considerations are discussed in greater detail below, where a correlation between hybridized state time and structural parameters is described.

3.3.4  *Melting Modes Correspond to Distinct Structural States of Association*

We hypothesized that the two observed modes of melting, with characteristic time scales of 1.4 s and 15 s respectively, might correspond to distinct states of association. Accordingly, the trajectory data were mined to identify possible correlations between the mean value of $d$ for a given hybridized state and the time interval of that hybridized state. These data are presented in Figure 3.9 in the form of a heat map. It is visually apparent that there is a strong correlation between hybridized states with small values of $d$ and states with short time intervals. For example, the vast majority of hybridization events with $d<0.4$ exhibit state times shorter than 3 s, while hybridization events with $d>0.4$ often remain hybridized for much longer time intervals.

To make this distinction even more apparent, Figure 3.10 shows cumulative hybridized state time distributions for two populations that are apparent in Figure 3.9 as ranges of $d$ ($d<0.4$ and $0.4<d<1.2$), and fit with a number of exponentials selected by maximum entropy method. As expected from the heat map in Figure 3.9, the two distributions varied significantly, with the small $d$ distribution decaying much more rapidly. The small $d$ distribution was well-described
by a single exponential with a characteristic melting time of $1.9 \pm 0.3$ s, similar to that of the fast melting mode determined from the overall hybridized state time distribution. The large $d$ distribution was described by a sum of two melting modes, including a dominant mode with a characteristic time constant of $16.3 \pm 0.4$ s, similar to that of the slow mode determined from the overall hybridized state time distribution. The large $d$ distribution also exhibited a small fraction of melting events with a characteristic time constant of $\sim 0.1$ s, which was attributed to the dissociation of non-specific interactions as discussed earlier. Thus, the two melting time constants that were determined from fitting the overall hybridized state time distribution can

Figure 3.9 - Heat map of state time \textit{versus} mean $d$ for every observed hybridization event. The legend to the right depicts color associated with counts of molecules in each bin.
attributed to distinct populations that were identified on a structural basis, i.e. their mean \(d\) value. While this correlation is interesting, the specific details are initially somewhat surprising, since one might intuitively expect that hybridization events that exhibit small values of \(d\) might be more strongly associated and remain associated longer; instead, the opposite was observed.

Clearly the mean \(d\) value associated with a given apparent hybridization event is not necessarily the only relevant structural parameter. In particular, one might expect that structural fluctuations might influence the likelihood of melting. Therefore, the trajectory data were analyzed to identify possible correlations between the mean value of \(d\) for a given hybridized state and the variance in \(d\) during that hybridized state (as a measure of the degree of
fluctuations). These data are presented in Figure 3.11 in the form of a heat map. Interestingly, the data clearly show that hybridized states with small mean values of $d$ exhibit significantly larger fluctuations than hybridized states with larger values of mean $d$. This is again somewhat counter-intuitive structurally, but totally consistent with the fact that the large $d$ population is much longer-lived than the small $d$ hybridized states.

As discussed earlier, based on our estimates of the Förster radius and the molecular geometry, a nominal hybridized state is expected to have a mean value of $d=0.9$ and a variance $\sigma^2=0.1$. Therefore, it was hypothesized, that the large $d$ population, which is long-lived ($\sim 15$ s)

Figure 3.11 - Heat map of variance in $d$ versus mean $d$ for every observed hybridization event. The legend to the right depicts the color associated with counts of events in each bin.
corresponds to this nominal hybridized state. The appearance of two modes of melting, from two distinct states, was intriguing and somewhat unexpected. In attempt to characterize the driving forces behind this phenomenon, several hypotheses were proposed. For example, the short “hybridization” times, low \( d \)-values, and large variance associated with the false hybridization mode could possibly be explained by non-specific interactions between just the donor and acceptor fluorophores. However, this hypothesis was inconsistent with the results of the negative control experiments using the non-complementary poly-A probe DNA, which did not exhibit this behavior. In particular, the poly-A control exhibited only a single extremely short-lived “hybridization mode” (~200ms) in contrast with the characteristic state time of ~2s for the “false” hybridization mode of the complementary probe. Alternatively, it is possible that the false hybridization mode could represent a hybridization state where the molecule is periodically zipping and unzipping the base pairs near the end of the strands. This hypothesis satisfies the observed high variance in the short hybridization mode, but fails to account for close average proximity of fluorophores indicated by the low mean value of \( d \) (high \( E \)) in this mode. A final possibility for the false hybridization mode involves an alternative specific secondary structure, e.g. mismatched hybridization or another mode of coordination. It is difficult to picture how this would lead to extremely close association of fluorophores (i.e. small \( d \)); however, it cannot be ruled out entirely as a possibility. A structural picture of the small \( d \) population is somewhat difficult to define, and we hesitate to speculate too broadly. What can be said with certainty is that this population represents associations between complementary ssDNA, in the near surface region, that exhibit a small mean donor-acceptor distance with large
fluctuations. This leads to much shorter contact times than observed for the nominal hybridized state.

3.4 Conclusions

Single molecule tracking has provided a unique opportunity to probe the effects of surface chemistries on the pathway to hybridization in an environment that is relevant to nucleic acid assays such as DNA microarrays. Unique information is accessible, providing insight into the presence of distinct molecular populations and pathways associated with hybridization and melting in the near-surface region. The experiments performed here revealed that surface-mediated DNA hybridization occurred via a search process that involved a complex combination of two- and three-dimensional mobility. Furthermore, information was obtained about the kinetics of melting and re-hybridization. The presence of these competing processes suggested that surfaces may be defined to optimize hybridization efficiency and the stability of the hybridized state, and the data presented here provide initial design principles for such surfaces.
3.5 References


(9) Kastantin, M.; Schwartz, D. K., Small 2013, 9, (6), 933-41.


3.6 Supporting Information

Single-molecule total internal reflection fluorescence microscopy (TIRFM) in conjunction with resonance energy transfer (RET) was used to observe the dynamic behavior of ssDNA at the interface between aqueous solution and solid surface covalently modified with complementary ssDNA via epoxide-amine chemistries. The use of RET provides the ability to probe the spatial distance between proximal complementary strands and was used as an indicator of hybridization. This approach permitted the extraction of state time distributions for the “hybridized” state and “searching” state over a range of temperatures. Control experiments were also performed using a poly-A oligonucleotide instead of the complementary poly-AC strand. Due to the large volume of data obtained in these experiments, some details were omitted from the main text of the manuscript. Therefore, additional information is presented here, including the relative distance between donor and acceptor fluorophores, \( d \), for the negative control (Figure 3.12), sensitivity analysis of the hybridization threshold (Figure 3.13), complete temperature data for the fractional analysis of transitions between states (Figure 3.14), the comparison of poly-AC and poly-A behavior in state time distributions (Figure 3.15), all the characteristic state times and their respective population fractions for this study (Table 3.1), Arrhenius analysis of the characteristic surface melting times (Figure 3.16), the structures of both the poly-TG probe and poly-AC target oligonucleotides (Figure 3.17), and the data used to determine the optimum epoxysilane (GPTMS) deposition conditions (Figure 3.18).

3.6.1 Relative Distances Between Donor and Acceptor Fluorophore Distribution for Poly-A Control
As described in the main text, criteria to distinguish between hybridized oligonucleotides and oligonucleotides in the “searching” state were established using the relative distance between donor and acceptor fluorophores, $d$. The relative distance was calculated for all observed trajectories in both the control and complement target molecule trials. In Figure 3.12, step-functions were used to describe extreme $d$-values. Values of $d$ below 0.7 were considered to show complete-RET and make up an extremely small fraction of total observations. Zero-RET observations are shown as the step function for $d>2.0$, indicating that a large fraction of poly-A strands engage in negligible RET interactions. Examination of the distribution indicates the presence of two populations, one centered at $d=1.5$, indicating weak RET, and a second showing zero RET, $d>2.0$. This suggests that the $d=1.5$ populations are a result of incidental intermolecular interactions between the control donor-labeled target and the non-complementary immobilized acceptor-labeled probe. This observation, along with analogous

![Figure 3.12](image)

Figure 3.12 - Relative distances between donor and acceptor fluorophore distribution for all poly-A control species observed to undergo RET in experiments at 293K.
data for the complementary donor-labeled target (Figure 3.2), suggested that $d=1.6$ was an appropriate criterion for assigning hybridization events.

### 3.6.2 Sensitivity Analysis of Hybridization Threshold

In the main text of the article the relative distance between donor and acceptor fluorophores was used to identify when hybridization events occurred. The selection criteria involved a cut-off where steps of a trajectory with $d$ values less than the threshold were considered to be hybridized and values greater than the threshold were searching. It was important when selecting these criteria to choose a hybridization threshold that was low enough to avoid selecting noise or incidental RET events as hybridization events, while not being low enough to identify partial melting events or other fluctuations in RET signal as true dehybridization events.

To insure that a sufficiently high hybridization threshold was selected, data was mined and a hybridization threshold sensitivity analysis was performed. This analysis extracted the number of observations of apparent “fluctuations” where a molecule rapidly crossed over the threshold and returned to the hybridized state. The threshold was systematically increased from $d = 0-100$ ($E = 1 - 10^{-12}$) and the number of fluctuations calculated. The results of this sensitivity analysis are plotted in Figure 3.13. The fluctuations that exist at large threshold values are due to the tracking of species that exhibit little RET, or $d$ values approaching infinity, alternating with weak RET signals attributed to noise. In the range of $d = 1.6 - 2.0$ ($E = 0.06 - 0.02$) fluctuations vary by less than 10% from the number of fluctuations at $d \geq 10$ ($E \geq 10^{-6}$). This information combined with the results stated in the main text indicated that a hybridization
threshold of \( d = 1.6 \) would reduce identification of false melting events, incidental RET, and RET signals due to noise.

3.6.3 Temperature Dependence of Fractional Pathways to Hybridization

Molecular trajectories were examined and then partitioned into “searching” and “hybridized” states based on RET efficiency. Transitions between states could then be counted and normalized by the destination or source state. These fractions were then assembled to provide a picture of the pathway to surface hybridization. This analysis was repeated for the temperature range of 293K – 313K at 10 degree intervals; results are shown in Figure 3.14. The fractions were insensitive to temperature in this range. This observation was statically confirmed as described in the manuscript.
3.6.4 Poly-AC and Poly-A State Time Distributions

Cumulative state time distributions were prepared by monitoring the dynamic behavior of single-molecule RET trajectories. Using the relative distance between fluorophores “hybridized”- and “searching”-state time intervals were accumulated and used to create cumulative state time distributions. This process was repeated for both the poly-A and poly-AC oligonucleotides. The resulting distributions are compared in Figure 3.15.
3.6.5 Characteristic State Times as a Function of Temperature

State time distributions were prepared for all transitions at each measured temperature. These distributions were fit with a number of exponentials selected via the maximum entropy method as described in the text. Hybridization from the searching state had only a single characteristic search time, $\tau_{s,\text{on}}$. While there were multiple characteristic state times for desorption from searching state, $\tau$, desorption from hybridization, $\tau_{\text{des,off}}$, and melting to the surface, $\tau_{s,\text{off}}$. These characteristic residence times and their respective population fractions, $f$, are given in Table
3.1. As discussed in the text the majority of characteristic state times show little temperature dependence. However, the melting state times exhibit systematic variation. The longer hybridization state has a strong temperature dependence indicated by the decreasing characteristic state times from 15s to 8s over the range of 293K-313K. On the other hand, the shorter hybridization state demonstrates little temperature dependence. This difference is elaborated upon in the primary text.

Table 3.1 - Characteristic state times, $\tau$, and their associated population fractions, $f$, for the exponential fits of all state time distributions over the temperature range 293K – 313K. The numbers in parentheses represent the uncertainty in the least significant digit.

<table>
<thead>
<tr>
<th>Mode</th>
<th>293 K</th>
<th>303 K</th>
<th>313 K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$</td>
<td>$f$</td>
<td>$\tau$</td>
</tr>
<tr>
<td>$\tau_{s,\text{on}}$</td>
<td>0.110(6)</td>
<td>1</td>
<td>0.106(2)</td>
</tr>
<tr>
<td>$\tau_{s1,\text{off}}$</td>
<td>1.47(3)</td>
<td>0.55(2)</td>
<td>1.44(3)</td>
</tr>
<tr>
<td>$\tau_{s2,\text{off}}$</td>
<td>15(1)</td>
<td>0.45(2)</td>
<td>11.2(2)</td>
</tr>
<tr>
<td>$\tau_{\text{des1,off}}$</td>
<td>0.122(7)</td>
<td>0.28(1)</td>
<td>0.123(4)</td>
</tr>
<tr>
<td>$\tau_{\text{des2,off}}$</td>
<td>1.4(2)</td>
<td>0.22(1)</td>
<td>1.6(3)</td>
</tr>
<tr>
<td>$\tau_{\text{des3,off}}$</td>
<td>15.1(8)</td>
<td>0.50(1)</td>
<td>10.8(6)</td>
</tr>
<tr>
<td>$\tau_{1}$</td>
<td>2.2(1)</td>
<td>0.94(4)</td>
<td>2.0(4)</td>
</tr>
<tr>
<td>$\tau_{2}$</td>
<td>0.20(1)</td>
<td>0.06(2)</td>
<td>0.17(2)</td>
</tr>
</tbody>
</table>
3.6.6  *Arrhenius Analysis*

In the primary manuscript the temperature was systematically varied from 293K – 313K at intervals of 10°K. For each temperature, state time distributions were extracted and fit with a multi-exponential fit. The characteristic state times (τ) and their associated population fractions were extracted from these fits. Hybridized state times correspond to the time spent hybridized before melting. The hybridization state times demonstrated strong temperature dependence. The characteristic times were inverted to represent an apparent rate of melting and then plotted on an Arrhenius plot (Figure 3.16). The trend was fit using standard regression analysis; the results of these fits are reported in Figure 3.16 in the form of the natural log of the Arrhenius equation. The Apparent activation energies were $E_a = 23$ kJ/mol and $E_a = 22$ kJ/mol for melting to the surface and into solution, respectively. These values are somewhat smaller

![Arrhenius plots for duplex DNA melting to the surface and into solution.](image)

Figure 3.16 - Arrhenius plots for duplex DNA melting to the surface and into solution.
than the analogous activation energies expected for DNA melting behavior in solution and are discussed in more detail in the main body of the text.

3.6.7 Structure of Target and Probe Oligonucleotides

The structures of both the poly-TG probe and poly-AC target oligonucleotides are shown in Figure 3.17. The linker/fluorophore combinations are drawn explicitly and the DNA sequences are given by their letter code. The two sequences are arranged to resemble the structure of the molecules during hybridization. As discussed in the text the poly-AC target strand is labeled with a C6-Amino/Alexa Fluor 488, linker/fluorophore combination on the 5’ end. The 3’ end of

Figure 3.17 - Structure of labeled probe and target ssDNA.
the poly-AC molecule is unmodified. The 5’ end of the poly-TG complementary probe molecule was modified with a C6-Amino linker. A C7-Amino/Quasar 670, linker/fluorophore combination was attached to the 3’ end.

3.6.8 Optimized GPTMS Monolayer Deposition Conditions

Vacuum assisted chemical vapor deposition was performed on silicon wafers (WRS). The deposition solution was a 1:2:20 solution by volume, of n-butylamine:3-glycidoxypropyltrimethoxysilane (GPTMS):toluene. The solution was placed in the bottom of a desiccator; silicon wafers were mounted in the desiccator via Teflon holder. The desiccator was sealed and evacuated via house vacuum and left to incubate for times ranging from 0-24 hours. Samples were extracted from the deposition chamber and immediately characterized using sessile drop experiments on a contact angle goniometer and variable angle spectroscopic ellipsometry. The results of these analyses are shown in Figure 3.18. Ellipsometry was used to measure film thickness. The measured film thickness was compared to the length of a fully extended GPTMS molecule film (dotted line, Figure 3.18); thicknesses equal to this value indicate the presence of a complete monolayer. Contact angle goniometry was used as a complementary technique. The contact angle quantifies the wettability of the surface and is sensitive to different surface chemistries and can be used to characterize a complete monolayer. The combination of these two techniques allowed for the optimization of the deposition of high quality GPTMS monolayers.
Figure 3.18 - Film thickness (Å), left axis, was measured using variable angle spectroscopic ellipsometry (◊). The dotted line indicates the length of a fully extended GPTMS molecule.
Chapter 4: DNA Utilizes Forager Behavior to Search for a Binding Partner

4.1 Introduction

Search processes play a significant role in ecological\textsuperscript{1-3}, military\textsuperscript{4,5}, and molecular systems that comprise applications in (bio)chemical reactions\textsuperscript{6,7}, chemical sensing and bioassays\textsuperscript{8,9}, signal transduction in biomembranes\textsuperscript{10,11}, etc.\textsuperscript{12,13}. These systems all encounter the same challenge: what strategy should a searcher employ to find target sites efficiently? Effective searches are comprised of several components, including the searcher’s knowledge, range of detection, search rate, target density, and search strategy\textsuperscript{14}. While some organisms employ knowledge or active detection as part of searching (e.g. chemotaxis), foraging generally refers to the type or phase of searching that is knowledge-free. Moreover, a foraging search is considered to be complete when the searcher comes within the detection range of its target\textsuperscript{5}, e.g. where a submarine hunter actively senses a target using sonar or a predator visually detects its prey. The subsequent “capture” process relies on different strategies\textsuperscript{1-3}. In molecular systems, it is generally assumed that the search is knowledge-free and the detection range is defined by the so-called reaction radius ($\alpha$). The rate of the search is defined by the speed with which the predator hunts its prey (distance/time) or the diffusion coefficient ($D$) of a molecule searching for its target (area/time). Of course, the search is universally minimized for the greatest search speed (or diffusion coefficient)\textsuperscript{15} that is accessible to the searcher. Based on these considerations, and on the target density, a searcher will adopt different searching strategies/algorithms.
The Lévy-flight foraging hypothesis makes predictions based on target density, which is related to the starting distance between searcher and target \((R)\)^{16}. Specifically, the hypothesis predicts that in a system where targets are sparse, searchers should adopt a strategy of alternating short-range searches (Lévy-walks) and longer (non-searching) Lévy-flights\(^{17-19}\). This so-called continuous time random walk (CTRW) process uses step-lengths drawn from a Pareto-Lévy distribution\(^{20-22}\). When targets are abundant, Brownian motion is a sufficiently effective strategy\(^1\). However, in ecological, physical, and chemical systems, theory predicts that Lévy flights are often a more efficient search strategy than Brownian behavior for locating randomly distributed targets\(^{14, 23}\).

Interestingly, recent observations by our group and others suggest that the behavior of a wide variety of surface-adsorbed molecules mimics the same type of motion favored for sparse prey searches\(^{24-26}\). In particular, proteins, polymers and small molecules all exhibited intermittent motion which corresponds to a continuous time random walk with periods of slow local diffusion (or even apparent immobility) alternating with long flights that were approximately power-law distributed. These observations were consistent with longstanding theoretical models\(^{27-31}\) that predict similar phenomena due to “desorption-mediated diffusion”, \textit{i.e.} the notion that molecules move on surfaces via a sequence of desorption, three-dimensional walks through an adjacent liquid phase and subsequent re-adsorption. This coincidental similarity between molecular motion at interfaces and forager dynamics suggests that molecular searching may be similarly enhanced under sparse target conditions.
Many models have been developed to describe forager dynamics\textsuperscript{1,15,32-34}. To directly test these models, in this paper, a system involving molecular searching in two-dimensions (2D) is described. Two models, both developed by Benichou and co-workers, were selected to calculate the mean first passage time for comparison to our experimental system\textsuperscript{15,31}. The first model describes a purely Brownian search behavior at the solid-liquid interface. The second model describes an intermittent Lévy-flight search process. In the latter model, a searcher adsorbs to a surface and freely diffuses in 2D for a stochastic “waiting time”. The searcher then desorbs into the adjacent liquid phase, were a non-searching flight (which can be very long) is executed before reabsorbing. This alternating process is continued until the searcher finds a target. By comparing the predictions of these models to the measured search times of DNA, we have been able to identify the search strategy used by DNA at the solid-liquid interface.

Specifically, we have performed high-throughput dynamic single molecule (SM) studies of ssDNA hybridization at solid-liquid interfaces. A chemical immobilization method was developed to tether fluorescently-labeled DNA at a well-defined surface density within a matrix of controlled hydrophobicity. In contrast with conventional characterization methods, which provide ensemble averages and/or steady-state information, single-molecule tracking separates and identifies competing mechanisms, by providing detailed information about large numbers of molecular trajectories (>600,000 individual trajectories were analyzed here). Total internal reflection fluorescence microscopy (TIRFM)\textsuperscript{35} was used in conjunction with intermolecular Förster resonance energy transfer (FRET) and alternating laser excitation, allowing direct quantitative analysis of molecular search behavior as a function of the initial searcher-target
These search times were directly compared to theoretical predictions based on Brownian searching and intermittent Lévy-flight searching respectively.

4.2 Experimental Details

4.2.1 Surface Preparation

Fused silica (FS, Mark Optics) wafers were washed in a 2% Micro 90 solution and manually scrubbed with a Kim-wipe prior to rinsing with ample amounts of Milli-Q water. The wafers were then rinsed with isopropanol and dried with ultrapure nitrogen. The wafers were subsequently placed in a piranha solution (3:1 sulfuric acid:hydrogen peroxide) at approximately 70 °C for 1 h followed by copious washing with Milli-Q water and drying with ultrapure nitrogen. The dry wafers were then treated with UV-ozone for 1 hour. After the UV-ozone cleaning, a 1:2:20 solution by volume, of n-butylamine:3-glycidoxypropyltrimethoxysilane (GPTMS, 95% pure, Gelest):toluene was used to deposit a monolayer on the FS wafers via vacuum-assisted vapor deposition for 16-20h. Surfaces were further modified with either zinc-thiolate-disulfide or lithium hydroxide catalyzed thiol-epoxide chemistries. Hydrophobic surfaces were created by incubating GPTMS modified fused silica in a mixture of 1-(pentyldisulfanyl)pentane (0.5 mmol, Aldrich), zinc powder (2 mmol, Fluka), and aluminum chloride (1.2 mmol, 99.99% trace metal basis, Aldrich) suspended in DMF (10 mL) for ~18 hours at 65 °C. Hydrophilic surfaces were created by incubating GPTMS modified fused silica in a solution of 100mg Methyl-PEG₄-Thiol (Thermo Scientific) with 100mg lithium hydroxide (≥99.998% metal basis, Fluka) in 100ml DMF for ~24 hours. Probe molecules were attached to the surface by placing either a disulfide or thiol linker at the 5′ end of 3′-
TGTTGGTTGTGTGGT-5’ (poly-TG, Biosearch) DNA labeled with Quasar® 670 dye and adding enough DNA to make a 1nM solution in either incubation techniques. The modified surfaces with covalently attached were exposed to solutions containing the fluorescently labeled complementary DNA 5’-ACAACCAACACACCA-3’ (poly-AC, Invitrogen) with the 5’ end modified with AlexaFluor® 488 dye via C6-Amino linker at 100pM concentrations in 1x PBS buffer.

4.2.2 Contact-Angle Measurements

Contact angles (CA) of all surfaces were measured with a custom-built contact-angle goniometer. A 1 μl drop of Milli-Q water was deposited on the surface in seven random locations on three separate samples; the averaged values and standard deviations were reported. For unmodified FS samples, almost complete wetting of the surface was observed (CA < 5°). The CA for GPTMS was 54° ± 3° corresponding to a complete monolayer, measured by ellipsometry.17,21

4.2.3 Total Internal Reflection Fluorescence Microscopy Measurements

A custom-built prism-based microscope comprised of a Nikon TE-2000 microscope with a 60x water immersion objective was used to preform total internal reflection fluorescence microscopy (TIRFM) measurements. The intensity of the illumination was adjusted to permit single fluorophore observation with a 100 ms acquisition time during sequential imaging, while also allowing continuous observation for several minutes without photobleaching.22 Movies were 1200 frames in length (120s), six movies were taken per sample, and experiments were
run in triplicate. An Optosplit II image splitter (Cairn Research) was used for dual channel imaging. This device split the image via a 610 nm dichroic mirror; the dual images were then filtered by band pass filters optimized for the AlexaFluor®-488 and Quasar®-670 dyes. Donor bleeding and direct acceptor excitation were effectively eliminated through filter selection.¹⁷ Images were aligned during post-processing by convolving the two channels as described previously by Kastantin et al.¹⁸ Both poly-AC complementary target ssDNA and poly-A control ssDNA experiments were observed at temperatures 293K, 303K, and 313K using a temperature controlled stage. All objects that resided on the surface for less than one frame (100 ms) were removed from the data pool, as it was impossible to accurately measure state times of objects that resided on the surface for less than one frame.

4.2.4  Resonance Energy Transfer

Spatial information in fluorescence imaging was acquired via resonance energy transfer (RET). This technique involves the non-radiative energy transfer from a donor fluorophore (Alexa-488) to an acceptor fluorophore (Quasar-670) that occurs when the two fluorophores are within 1-10nm,²³ where the specific distance-dependence is characteristic of the specific RET-pair is defined by the Förster radius \( R_0 \).²³ This radius is the distance where the non-radiative energy transfer between donor and acceptor fluorophores is 50%.²⁴ The Förster radius for the Alexa-488/Quasar-670 RET pair is approximately 5.4 nm, the exact value is dependent on the molecular orientation and medium conditions.²⁵ The Förster radius coupled with the experimentally observed fluorescence intensity of the donor \( F_D \) and acceptor \( F_A \) can be used
to calculate the relative separation of the RET pair. This relative distance between donor and acceptor fluorophore \(d\) was used to characterize DNA hybridization events.\(^{18}\)

\[
d = \left( \frac{F_D}{F_A} \right)^{1/6} \tag{3}
\]

The relative distance \(d\) is directly related to the RET efficiency through the expression:

\[
E = \frac{1}{1 + d^6} \tag{4}
\]

While the RET signal can equally well-described using either \(d\) or \(E\), we have chosen to express our data in terms of \(d\). As a quantity that is proportional to the physical donor-acceptor separation, \(d\) provides a degree of physical intuition not given by \(E\).

### 4.2.5 Data Analysis

Diffraction-limited objects were identified in each channel on a frame-by-frame basis using a disk matrix and thresholding algorithm.\(^{26}\) For each frame, both channels were convolved to identify objects in the same position. An object’s location was identified using center-of-intensity calculations. The total fluorescence intensity of each object was determined by integrating the intensities of all pixels associated with the object, and subtracting the local median background intensity. An object that appeared within 3 pixels (681nm) in consecutive frames was identified as the same object for purposes of tracking. Objects simultaneously identified within 2 pixels (455 nm) in both donor and acceptor channels was identified as a RET event. The position of a RET event was identified using the channel with greatest signal to noise ratio. Fluorescence intensity was monitored in both channels for each molecular trajectory.
These intensities were used to calculate the relative distance between fluorophores, $d$, using eq.1 on a frame-by-frame basis.

In cases of extremely strong or extremely weak energy transfer, the intensity of a given object was high in one channel but below the noise threshold in the opposing channel. In these cases, the position of the identified object from the strong channel was applied to the weak channel to determine the effective intensity of the object in the weak channel. At least 100,000 molecular trajectories were obtained for the target and control molecule on the surface. Only objects that were directly observed to both adsorb and desorb were used in the analysis to reduce uncertainty with respect to the surface state times. A given surface state time of each object (e.g. searching or hybridized) was calculated as the number of frames in which the object was identified in that state multiplied by the acquisition time (100 ms). Since an object may not reside in the initial and/or final frames for the entire exposure time of that frame, the uncertainty in a given state time interval was taken to be the exposure time divided by $\sqrt{2}$.

4.2.6 Identification of searching and hybridized states

Hybridized oligonucleotides were distinguished from searching oligonucleotides using an appropriate criterion (a “threshold”) based on FRET efficiency ($E$) or, equivalently, a sufficiently low value of the relative donor-acceptor distance, $d$. In order to verify the presence of these distinct states, and to establish appropriate criteria for distinguishing states, statistical distributions of all observed $d$ values were created for all trajectories in a given experiment (Extended Data, Figure 4.10). Based on these observations, a criterion was defined where the
threshold between the searching and hybridized states was defined as \( d = 1.6 \). The nature of this selection and its impacts on the data set have been discussed in detail previously. Though the selection of a hybridization threshold was somewhat arbitrary, a different threshold choice results only in small changes to some numerical values reported above (i.e., melting times); the main conclusions of this paper were found to be robust with respect to small variations in the hybridization threshold criterion.

4.2.7 Search Success Fraction

The search efficiency of molecules was calculated by selecting all molecules at a starting distance from their target and calculating the percentage of molecules that succeed at finding a complementary strand. The necessary data was acquired by directly exciting the acceptor modified DNA covalently attached to the surface to identify target locations. This was immediately followed by the standard TIRFM procedure. A 100 nm wide annulus centered each target location was used to identify all trajectories at starting distances of 200 nm, 400 nm, 600 nm, 800 nm, 1000 nm, 1200 nm, and 1400 nm.

4.2.8 Mean First Passage Times

To calculate mean first passage times, molecular trajectories that succeed at locating a complementary strand were selected from the data set. The history of these trajectories was analyzed to identify the point of adsorption and the point of hybridization. The distance between these two points was calculated to find the "starting distance."
were selected for 100 nm, 500 nm, 1000 nm, and 1500nm with a bandwidth of 100nm. For example, molecules with a starting distance of 500 nm, only use trajectories with starting distances ranging from 450-550 nm. The first passage time was calculated by counting the number of steps and molecule took before hybridizing multiplied by the aquisition time (100 ms). These values were then accumulated and the mean first passage time and standard error were calculated.

4.2.9 *Squared-Displacement Distributions*

The squared-displacement was calculated for each step of each trajectory. Experimental cumulative squared-displacement distributions were created by sorting the squared-displacement data and ranking each data point. The error for each data point in the distribution represents a 68% confidence interval for a Poisson distribution. The cumulative distribution was then fitted to a Gaussian mixture model to extract population fractions and their respective diffusion coefficients. A more detailed discussion of this analysis was given previously. Mean diffusion coefficients were calculated by the weighted average of fitted coefficients. The data for diffusion coefficients was collected in the absence of target DNA.

4.2.10 *Residence Time Distribution*

The surface residence time of each object was calculated as the number of frames in which the object was identified multiplied by the exposure time (100ms) to convert to units of time. Since an object may not reside in the initial and/or final frames for the entire exposure time of that
frame, the uncertainty in the residence time was taken to be the exposure time divided by \( \sqrt{2} \).
The cumulative residence time distribution was modeled using a multi-exponential function, where the coefficient of each term represents a population fraction and the time constant represents the characteristic residence time of that population. The error for each data point in the distribution represents a 68% confidence interval for a Poisson distribution. Mean residence times were calculated by a weighted average of the residence times of all observed populations. A more detailed discussion of this analysis was given previously\(^{51}\).

4.2.11 Search Radius Distribution

The search radius of each trajectory was calculated as the radius of gyration of that trajectory:

\[
R_g = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (r_i - \bar{r})^2} \tag{5}
\]

where \( N \) is the number of locations sampled in the trajectory, \( r_i \) is the position of step \( i \) in the trajectory, and \( \bar{r} \) is the mean position of the trajectory. The cumulative search radius distribution was modelled with an exponential mixture model, where the coefficient of each term represents a population fraction \( f_i \) and the length constant represents the characteristic search radius \( R_i \) of that population (equation \(6\)).

\[
p(t) = \sum_i f_i e^{-R/R_i} \tag{6}
\]
The error for each data point in the distribution represented a 68% confidence interval for a Poisson distribution. Mean search radii were calculated by a weighted average of the search radius of all observed populations.

4.2.12 Waiting Time

Experimental waiting times were measured by defining a one-step distance threshold of 0.15 μm. Step lengths in a trajectory that exceeded this threshold were considered to be a flight\textsuperscript{24}. The waiting time was calculated by counting the number of steps taken prior to a flight multiplied by the acquisition time. The cumulative waiting time distributions were assembled and modeled using an exponential mixture model, where the coefficient of each term represented a population fraction and the time constant represented the characteristic waiting time of that population. Mean waiting times were calculated by a weighted average of the waiting times of all observed populations.

4.2.13 Cumulative Melting Distribution

Cumulative state time distributions were created by accumulating trajectories and examining the time spent hybridized. Time steps in the hybridized state were identified by a high RET efficiency (\textit{i.e.} low \(d\)). The number of time steps taken before complementary strand melting was multiplied by the acquisition time (100 ms) to determine the melting time interval. The error for each data point in the distribution represents a 68% confidence interval for a Poisson distribution. The cumulative state time distributions were modeled using a multi-exponential
function, where the coefficient of each term represented a population fraction ($f_i$) and the time constant represented the characteristic state time ($\tau_i$) of that population.

4.3 Results and Discussion

Fused silica wafers were initially modified with epoxide-silane chemistry, and subsequently modified with either zinc-thiolate-disulfide$^{38}$ or lithium hydroxide catalyzed thiol-epoxide chemistry$^{39}$ to covalently attach DNA to the surface along with either a “hydrophobic” (2-(pentyldisulfanyl)pentane) or “hydrophilic” (methyl-PEG4-thiol) moiety. Sessile drop experiments were used to measure water contact angles on each surface to assess hydrophobicity$^{40}$. Surfaces modified with 2-(pentyldisulfanyl)pentane exhibited a contact angle of $70^\circ \pm 2^\circ$ indicating a more hydrophobic character, while surfaces modified with methyl-PEG4-thiol were hydrophilic, with a contact angle of $20^\circ \pm 3^\circ$. Surfaces for negative control experiments were prepared by performing surface modifications in the absence of DNA.

Intermolecular FRET, non-radiative energy transfer from a donor (AlexaFluor® 488) to an acceptor (Quasar®-670) fluorophore occurring over 1–10 nm$^{41}$, was used to identify the timing and location of successful searches. In our experiments, surface-immobilized ssDNA was modified with an acceptor fluorophore. Surfaces prepared with and without immobilized DNA were exposed to aqueous solutions (1x PBS, pH = 7.4) of the donor-labeled complementary strand (or a non-complementary control strand). A more detailed discussion of the experimental design is described in the experimental details. Dual-channel image sequences of all surfaces were acquired, comprising more than 600,000 molecular trajectories for the
complementary strands; molecules that resided on the surface for 100 ms (1 frame) or shorter were removed from the analysis. Fluorescence intensities of molecules were monitored in both channels. Observation of donor emission above a selected threshold was assumed to indicate a hybridization event. A detailed discussion of the “hybridization” threshold is described in the experimental details.

In these TIRFM experiments, fluorophores were excited by an evanescent field that decayed exponentially over distances of ~100 nm. Therefore, only donor-labeled DNA molecules that had adsorbed to the interface were resolved (Figure 4.1A, first frame); un-adsorbed molecules in solution near the surface were highly blurred because of their rapid motion and contributed only to background fluorescence levels. Adsorbed molecules were observed to move stochastically within the field of view, exhibiting apparent intermittent (i.e. CTRW) motion involving alternating slow/confined walks and long flights (Figure 4.1A, second frame). Some of the molecules (~20-40% of trajectories) eventually located an immobilized complementary strand (target) and hybridized, causing acceptor emission via FRET (Figure 4.1A, third frame). The locations of immobilized DNA were identified by periodic direct excitation of acceptor fluorophores, permitting the calculation of the initial “starting-distance” between each adsorbing donor-labeled DNA molecule and the nearest immobilized complementary strand. A detailed derivation and discussion of this parameter is described in the experimental details.
The representative trajectories plotted in Figure 4.1B demonstrate the dynamic behavior of a molecular search where molecules alternate between Lévy-flights and slow confined walks. Casual observation of these trajectories indicates a deviation from Brownian behavior. To better quantify this observation, the distribution of complementary DNA displacements (taken from all molecular trajectories) on the hydrophobic surface was compared to a Gaussian distribution.

Figure 4.1 - (A) physical interpretation of observations. In all parts of the figure, donor fluorophores are represented in green and acceptor fluorophores in red. (B) Example trajectories of successful Molecular searches. Ring indicates starting distance of 0.5μm. First step of the trajectories (-) and the last step of the trajectories (→). (C) Step size distribution of DNA Molecules on a hydrophobic Surface (---). Gaussian distribution using the mean diffusion coefficient of DNA molecules on a hydrophobic Surface (→→).
distribution representing the same mean diffusion coefficient (Figure 4.1C). These data represent the probability that a molecule has moved a distance \( \Delta x \) along the \( x \) or \( y \) coordinate during time \( \Delta t \). Previous work by Skaug et al. performed a related analysis for a library of biomolecules at the solid-liquid interface\(^{24} \), demonstrating that these distributions are not Gaussian, as one would assume for a simple diffusion process, but are comprised of a narrow Gaussian peak and extended tails. In Figure 4.1C, the Gaussian distribution (blue line) is strongly peaked near the origin and suggests that steps longer than \(~1 \, \mu m\) should have negligible probability, whereas the experimental distribution (red line) exhibits heavy non-Gaussian tails that extend to flights as long as \( 4 \, \mu m \).

Notably, as shown in Figure 4.1B, where the final trajectory steps are colored red, hybridization was observed to occur at various stages of the intermittent motion, \( i.e. \) not only following a long flight, but also after various periods of local searching. These combined observations suggest that the molecular searching of DNA is non-Brownian and qualitatively similar to the search strategy described by the Lévy hypothesis.

4.3.1 Search Efficiency and First Passage Times

The ability to identify molecular association events on the basis of FRET efficiency was used to calculate the distribution of first passage times (the time interval between initial adsorption and successful hybridization) and the search success fraction as a function of the initial searcher-target distance \( R \). By directly exciting the acceptor fluorophores, the positions of target sites on the surface were identified, permitting determination of the initial distance to a target site for
each adsorbing molecule. The fraction of successful searches was calculated for trajectories beginning at various starting distances (±50 nm) from these target sites (Figure 4.2A). For example, trajectories beginning within an annulus spanning 950–1050 nm in radius were included in the analysis for the $R=1000$ nm starting distance. Unsurprisingly, both surfaces approached a search success fraction of unity for $R=0$ nm and the successful fraction decreased systematically with increasing $R$, with success becoming very rare for $R > 1200$ nm. Notably, the search success fraction declined much more rapidly with distance on the hydrophilic surface compared with the hydrophobic interface. For example, for $R=400$ nm, ~60% of searches were successful on hydrophobic surfaces, but only ~25% on hydrophilic surfaces. In previous work, we found that ssDNA surface dynamics were strongly influenced by the physicochemical properties of a surface, including hydrophobicity. These observations demonstrated that for short DNA lengths (< 25 bases long) diffusion was enhanced and surface residence times reduced on more hydrophobic surfaces. These observations were supported in this work as
seen in extended data, Figure 4.5 and Figure 4.6. We hypothesized that the difference in search efficiency on hydrophobic and hydrophilic surfaces may be related to the characteristic size of the region explored by a DNA molecule prior to desorption. To estimate this mean search radius, \( \bar{R} \), the radius of gyration was calculated for each trajectory, the cumulative search radius distributions were accumulated and fit with an exponential-mixture model (Extended Data, Figure 4.7). The characteristic search distances and their respective population fractions were extracted from these fits and used to calculate the weighted mean search radius, \( \bar{R} \). This method of calculating the mean of the distribution is preferable to calculating the arithmetic mean. Because the resolution limit of our experiments gives us only the tail of the distribution, by fitting the distribution we can account for behavior below our resolution limit. The mean search radii calculated in this way were 330 ± 50nm and 420 ± 60nm for hydrophilic and hydrophobic surfaces respectively. These values are shown as vertical lines in Figure 4.2A, which indicates a strong correlation between the mean search radius and the successful fraction. In particular, for each type of surface chemistry, the mean search radius represents the approximate distance at which 50% of searches were successful.

Using only trajectories associated with successful searches, the first passage times were calculated for trajectories with various starting distances \( R \). The mean first passage time (MFPT) is shown in Figure 4.2B for \( R = 100 \) nm, 500 nm, 1000 nm, and 1500 nm. The full first passage time distributions are included in extended data, Figure 4.8. The MFPT values were systematically smaller (by nearly a factor of two) on hydrophobic surfaces than on hydrophilic surfaces, consistent with the faster diffusion on hydrophobic surfaces as described previously.
Moreover, as expected, the MFPTs increased systematically with increasing $R$, ranging from ~0.4–1 s on hydrophilic surfaces and 0.2–0.6 s on hydrophobic surfaces.

4.3.2 Comparison of Experimental and Model Mean First Passage Times

The criterion described above allowed us to identify molecular association events on the basis of RET efficiency; this was further used to calculate mean first passage times and search success fraction as a function of starting distance from targets. To calculate the search success fraction all molecular trajectories were considered. By directly exciting the donor fluorophores it was possible to identify target sites on the surface. Trajectories beginning in a 100nm wide annulus with a radius equal to the “starting distance” around these target sites were selected for analysis. The fraction of successful searches was calculated for starting distances from 200nm – 1400nm for both the hydrophobic and hydrophilic surfaces (Figure 4.3A). Unsurprisingly, both surfaces approach a search success fraction of unity for a starting distance of 0nm. But as the starting distance approaches 1400nm the successful searches become more rare. It was interesting to see that DNA search success fraction on the hydrophilic surface rapidly declines with starting distance when compared with the hydrophobic interface. This suggests that DNA searching is enhanced on hydrophobic surfaces.

The mean first passage times (MFPT) were also calculated by selecting molecules at a specific starting distance. However, this analysis only required molecules with successful searches. The time a molecule takes from adsorption to first hybridizing is the first passage time of that molecule. This was calculated for each trajectory, for distances 100nm, 500nm, 1000nm, and 1500nm from target molecules. The mean and standard error of first passage times was
calculated and plotted in Figure 4.3B. We see that as the starting distance approaches 0nm the MFPT also approach 0s. In the other extreme, search times become longer the further a molecule is from its target. Comparison of the hydrophobic and hydrophilic surface revealed that for all starting distances, the MFPT was smaller on the hydrophobic surface. This indicates that the time required to find a target was reduced on the more hydrophobic surface.

The dependence of both searcher success fraction and MFPT were strongly influenced by the physicochemical properties of a surface. Specifically suggesting, that the more hydrophobic surface provides an environment benefitting the search strategy adopted by DNA molecules. The Lévy-flight foraging hypothesis suggests that in an environment where targets are sparse molecules will adopt a strategy of alternating Lévy-flight and Brownian behavior.\textsuperscript{6, 7} It was previously reported that ssDNA surface dynamics were strongly influenced by the physicochemical properties of a surface, including hydrophobicity.\textsuperscript{22} In particular, short chain ssDNA (<25 bases) exhibits faster diffusion coefficient and desorption rates on hydrophobic surfaces, potentially influencing the time intervals associated with 2-dimensional searches for a complementary strand. This behavior in the hydrophobic environment would lend itself to more frequent Levy-flights and faster diffusion during short ranged searches. This may enhance the efficiency of mixed mode search strategies.

\textbf{4.3.3 \textit{Comparison of Experimental and Model Mean First Passage Times}}

The dependences of the searcher success fraction and the MFPT on starting distance were strongly influenced by the physicochemical surface properties, suggesting that the more
hydrophobic surface provided an environment benefitting the search strategy adopted by DNA molecules. According to the Lévy-flight foraging hypothesis, under sparse target conditions (as in the experiments described here) intermittent search strategies exhibit improved efficiency \(^{12,13}\). To test this directly, we compared the experimental results with two theoretical models, both developed by Benichou and co-workers, that predict the mean first passage time (i.e. the average search time) using different physical assumptions.

The first model describes the case of continuous Brownian motion (BM-model), where a searcher performs regular short range displacements while searching for a target. The MFPT for this model, \(\langle T_{BM} \rangle\), is calculated by equation 1\(^{34}\),

\[
\langle T_{BM} \rangle = \frac{A}{2\pi\bar{D}} \ln \frac{R}{a} \tag{1}
\]

where \(\bar{D}\) is the mean diffusion coefficient, \(R\) is the initial distance between searcher and target, \(a\) is the reaction radius (i.e. the detection range), and \(A\) is the search domain size. To calculate \(\bar{D}\) the cumulative squared-displacement distributions (Extended Data, Figure 4.6) were fit with a Gaussian-mixture model. Characteristic diffusion coefficients and their respective population fractions were extracted from these fits and were used to calculate \(\bar{D}=0.209 \pm 0.001 \ \mu m^2/s\) for the hydrophilic surface and \(\bar{D}=0.375 \pm 0.001 \ \mu m^2/s\) for the hydrophobic surface. The reaction radius, \(a\), was estimated by the radius of gyration of the probe molecule, 0.54 nm.
The second model, associated with intermittent interfacial searching (LF-model), describes short rapid interfacial searches periodically alternating with long range flights, and the MFPT of this model, $\langle T_{LF} \rangle$, is given by equation 2\textsuperscript{15}.

$$
\langle T_{LF} \rangle = \frac{\left[ \frac{\sqrt{2}}{\alpha a} \right]^2 - 1}{X_v^2 + \frac{2}{\alpha^2} D_2 \tau_w Y_v} \left[ X_v - \frac{2}{\alpha^2} D_2 \tau_w Y_v \right]^{\frac{1}{2}}
$$

where $X_v$ and $Y_v$ are functions containing the modified Bessel functions $I_v(a, D_2, A, \tau_w)$ and $K_v(a, D_2, A, \tau_w)$. In this equation, $A$ and $a$ are the search domain size and reaction radius (as in equation 1), $D_2$ is the diffusion coefficient associated with the slow searching time intervals, and $\tau_w$ is the mean waiting time between flights.

To calculate $\tau_w$, we measured experimental waiting times by defining a distance threshold of 0.15 $\mu$m to distinguish large displacements (Lévy-flights) from smaller diffusive steps. A cumulative probability distribution of these waiting time intervals is shown in extended data, Figure 4.9. The distributions of waiting times were fit with an exponential mixture model; characteristic waiting times and their respective population fractions were extracted from these fits and used to calculate $\tau_w=0.307 \pm 0.001$ s for the hydrophilic surface and $\tau_w=0.192 \pm 0.003$ s for the hydrophobic surface. The other parameter required for this model is the surface diffusion coefficient associated with the slow searching intervals, $D_2$. As described in previous work, this surface diffusion was identified as the crawling or slow diffusion mode and extracted from cumulative squared-displacement distributions\textsuperscript{43,44}. This approach gave values of $D_2=0.044$.
± 0.001 \mu m^2/s and \( D_2 = 0.058 \pm 0.001 \mu m^2/s \) for the hydrophilic and hydrophobic surfaces, respectively.

In order to apply these equations to the measured data, it was necessary to adapt them mathematically to the specific experimental realization. In particular, equations 1 and 2 represent the integrated MFPT for all molecules that start within a circular search domain, \( i.e. \) with a starting distance in the range \( 0 - A \). Thus, the predicted MFPT for a specific starting distance, \( R \), is related to the derivative of these models with respect to distance. With these differentiated models in hand, all necessary parameters (\( D, D_2, \bar{\tau}_w \)) were determined from control experiments in the absence of target DNA, and used to calculate solutions to each

Figure 4.3 - Semi-logarithmic plots of mean first passage times for molecules starting searches from a starting distance from target for (A) hydrophobic (\( \bullet \)) and (B) hydrophilic surfaces (\( \bigcirc \)). Including the results of Brownian motion model (\( \rightarrow \)) and Intermittent Lévy-flight model (\( \leftarrow \)).
model with no adjustable parameters. The results of these calculations are shown as red lines in the center of the shaded region with the experimental MFPT values in Figure 4.3 for both the hydrophobic and hydrophilic surfaces. We emphasize that these theoretical predictions used experimentally-measured values as inputs, with minimal assumptions, and no adjustable parameters. A sensitivity analysis was performed on the Benichou LF-model to determine the parameter with the largest impact on the predicted values. The LF-model was found to be most sensitive to changes in diffusion coefficient $D_2$; a 5% change in $D_2$ resulted in a 4-4.5% change in the predicted mean first passage time. Therefore, the LF-model was calculated while varying the diffusion coefficients by a factor of 2. The results of these calculations are shown as the shaded region in Figure 4.3.

In all cases, it is obvious that the theoretically predicted rapid decrease in MFPT for small values of starting distance is not reflected in the experimental measurements. In fact, this is a trivial artifact of the experimental temporal resolution, which involves sequences of images captured at intervals of 0.1 s. Due to this non-zero acquisition time, the minimum measurable time for a trajectory is 0.2 s, and the MFPT is therefore biased towards larger values, particularly in the near-target regime. Thus, we do not expect good agreement between theory and experiment for the $R=100$ nm data.

Notably, the LF-model is in reasonable agreement with the measured MFPT data for both hydrophobic and hydrophilic surfaces with no adjustable parameters. In particular, the agreement is within a factor of two or less for all observed points except in the near target case.
(shaded region, Figure 4.3). This is remarkable agreement considering the very simple physical assumptions incorporated into the theory. For example, previous observations have found that the time intervals between long flights (searching or waiting times) exhibit heavy-tailed\textsuperscript{24,25} (e.g. power-law) distributions, consistent with a broad landscape of surface-binding energies. In contrast, for simplicity, the Lévy-flight model\textsuperscript{15} assumes a simpler decaying exponential distribution consistent with a single binding energy. Interestingly, the actual search times were even faster than predicted from a conventional Lévy-flight model for long starting distances.

Importantly, for both surface chemistries the values given by the BM-model exceed the experimental results by an order of magnitude or more. Thus, molecules identify their targets at least ten times faster than expected for molecules executing a simple random walk with the same apparent mean diffusion coefficient. The dramatic effect is essentially due to the highly non-Gaussian nature of the step-size distributions shown in Figure 4.1C and extended data, Figure 4.6. It is remarkable that this enhanced search process happens to duplicate similar strategies that have evolved throughout the biological world.

4.3.4 Melting Time Distributions

In addition to revealing the different mechanisms associated with surface searching, a detailed analysis of molecular trajectories also provided kinetic information about the dynamics of DNA denaturing/melting. In particular, each hybridization event was dynamic, and ultimately ended via a transition back to a searching state or desorption, the mechanisms of which were described in detail previously\textsuperscript{36}. Figure 4.4 shows cumulative probability distributions for the
time intervals associated with the hybridized duplex DNA state on both surfaces. These data represent the fraction of molecules remaining hybridized for time \( t \) or longer. On these semi-logarithmic plots, a straight line would indicate first-order kinetics; any observed deviation from this behavior therefore, suggests the presence of multiple populations with distinct characteristic state times. These distributions were clearly nonlinear on the semi-logarithmic axes, and exhibited long tails extending to time intervals of >6 s. By fitting the data with an exponential mixture model and comparing the results to a negative control (to identify a characteristic time associated with accidental collisions as opposed to hybridization) it was possible to extract the characteristic melting time for the nominal hybridization mode\(^{34}\).

Comparison of the two surfaces demonstrates that the duplex DNA was longer lived on the hydrophilic surface with a nominal characteristic melting time of ~15 s, compared to the hydrophobic surface where the nominal characteristic melting time was ~10 s. These observations, combined with the analysis of searching, suggest that for these DNA sequences, the hydrophobic surface resulted in faster and more efficient DNA searching but reduced the longevity of duplex DNA.

Figure 4.4. Cumulative melting state time distributions for hydrophobic (•) and hydrophilic (▫) surfaces
4.3.5 Conclusions

Single molecule tracking has provided a unique opportunity to probe the dynamic strategies by which molecules explore surfaces to identify targets in an environment that is directly relevant to nucleic acid assays and other DNA biotechnologies. While it is intuitively reasonable that molecules might explore surfaces via two-dimensional Brownian motion, in fact, we found that searches were completed more than ten times faster than would be accomplished via a Brownian search due to the intermittent nature of molecular motion which involves local searching alternating with long and fast solvent-mediated flights. This process exhibits a remarkable similarity to intermittent search strategies (forager dynamics) that have evolved in biological systems under sparse prey conditions. Additionally, we identified competing effects of surface hydrophobicity on DNA searching behavior and double stranded DNA stability suggesting the need for careful optimization of surface chemistry for a given application.
4.4 References


4.5 Extended Data

Figure 4.5 - Semi-log plots of the cumulative surface residence time distributions of probe DNA on hydrophobic (●) and hydrophilic (☺) surfaces. The table includes the characteristic residence times and their respective population fractions for each surface. All data for these results were collected in the absence of target DNA.
Figure 4.6 - Semi-log plots of the cumulative squared displacement distributions of probe DNA on hydrophobic (∗) and hydrophilic (○) surfaces. The table includes the characteristic diffusion coefficients and their respective population fractions for each surface. All data for these results were collected in the absence of target DNA.

<table>
<thead>
<tr>
<th>Surface</th>
<th>$f_1$</th>
<th>$D_1$</th>
<th>$f_2$</th>
<th>$D_2$</th>
<th>$\bar{D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilic</td>
<td>0.685(1)</td>
<td>0.290(1)</td>
<td>0.315(1)</td>
<td>0.044(1)</td>
<td>0.209(1)</td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>0.920(1)</td>
<td>0.403(1)</td>
<td>0.080(1)</td>
<td>0.058(1)</td>
<td>0.375(1)</td>
</tr>
</tbody>
</table>
Figure 4.7. Semi-log plots of the cumulative Search radius of probe DNA on hydrophobic (●) and hydrophilic (▫) surfaces. The mean search radii are annotated. All data for these results were collected in the absence of target DNA.
Figure 4.8 - Mean first passage times for molecules starting searches from a starting distance to target for both hydrophobic (•) and hydrophilic (▫) surfaces and their respective first passage time distributions.
Figure 4.9 - Semi-log plots of the cumulative waiting time distributions of probe DNA on hydrophobic (♦) and hydrophilic (▪) surfaces. The table includes the characteristic waiting times and their respective population fractions for each surface. All data for these results were collected in the absence of target DNA.
Figure 4.10 - Distribution of the relative donor-acceptor distance for all RET observations in experiments involving complementary DNA on hydrophilic (-) and hydrophobic (--) surfaces.
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