Published in final edited form as:

Langmuir. 2012 June 5; 28(22): 8446–8455. doi:10.1021/la301165a.

Thermostable DNA Immobilization and Temperature Effects on Surface Hybridization

Dongbiao Ge, Xin Wang, Keeshan Williams, and Rastislav Levicky*

Department of Chemical and Biological Engineering, Polytechnic Institute of New York University, Brooklyn, NY 11201

Abstract

Monolayer films of nucleic acids on solid supports are encountered in a range of diagnostic and bioanalytical applications. These applications often rely on elevated temperatures to improve performance; moreover, studies at elevated temperatures can provide fundamental information on layer organization and functionality. To support such applications, this study compares thermostability of oligonucleotide monolayers immobilized to gold by first coating the gold with a nanometer-thick film (an “anchor layer”) of a polymercaptosiloxane, to which DNA oligonucleotides are subsequently tethered through maleimide-thiol conjugation, with thermostability of monolayers formed via widely-used attachment through a terminal thiol moiety on the DNA. The temperature range covered is from 25 to 90 °C. After confirming stability of immobilization and, more importantly, retention of hybridization activity even under the harshest conditions investigated, these thermostable films are used to demonstrate measurements of (1) reversible surface melting transitions and (2) temperature dependence of competitive hybridization, when fully matched and mismatched sequences compete for binding to immobilized DNA oligonucleotides. The competitive hybridization experiments reveal a pronounced impact of temperature on rates of approach to equilibrium, with kinetic freezing into nonequilibrium states close to room temperature and rapid approach to equilibrium at elevated temperatures. Modeling of competitive surface hybridization equilibria using thermodynamic parameters derived from surface melting transitions of the individual sequences is also discussed.

1. Introduction

Nucleic acid films on solid supports are of commercial as well as fundamental interest.^{1–6} The applications and science of DNA monolayers are strongly coupled in technologies such as DNA microarrays, the use of which rests on basic understanding to improve reproducibility, to provide physical insight into processing steps such as post-hybridization washing, and to mitigate noisy signals that otherwise can render large fractions of measured information unusable (e.g. see 6–9). An important, but poorly understood, aspect of DNA monolayers is the difference between thermodynamics of hybridization on solid supports and those in solution.^{5–6, 9} Progress on this issue has been hindered in part by difficulty of adapting standard methods for study of hybridization thermodynamics, such as calorimetry and melting curve analysis, to the surface context.

A primary challenge in characterization of DNA films is structural rearrangement and/or loss of the immobilized “probe” strands under aggressive conditions, including at elevated temperatures. For example, although melting transitions of immobilized DNA duplexes can be used to analyze thermodynamics of hybridization at surfaces,^{10–17} these measurements place heavy demands on layer stability as they can require hours of exposure to temperatures

rlevicky@poly.edu.

that can approach 90 °C. Stability of DNA monolayers is also an issue for studies of hybridization kinetics, especially for competitive scenarios where elevated temperatures are expected to strongly influence equilibration time scales of multiplexed reactions in which many analyte “target” sequences compete for binding to the probes. The importance of temperature in multiplexed hybridization is indicated by the observation that microarray applications use temperatures of up to 70 °C.¹⁸

Fundamental studies of surface hybridization have most often relied on nucleic acid films prepared on gold supports^{19–27} which offer precisely tailorable surface chemistry and compatibility with a range of characterization methods including optical, electrochemical, and microgravimetric techniques. (e.g. see refs. 17, 28–35) Strategies for enhancing stability of DNA monolayers on these versatile supports have included use of surface-reactive moieties with stabilizing contacts between neighbors,^{19, 23} use of multidentate attachment,^{19–22, 24–27} and alteration of the immobilization chemistry.^{19, 26} Among these various approaches use of polymerized anchor films (AFs) on gold^{15, 20} as well as other supports,^{36–37} in which the nucleic acid molecules are “anchored” through a strong covalent bond to an irreversibly bound polymer coating, has typically produced very stable films.

The present study investigates DNA monolayers formed on poly(mercaptopropyl)methylsiloxane (PMPMS) anchor films on gold.^{20, 24} A modification of the original approach²⁰ is described that, in addition to resistance to desorption, provides excellent preservation of hybridization activity. The key to this preservation is thorough locking-in of the PMPMS organization through crosslinking. A systematic comparison of stability is performed between PMPMS-anchored and widely used monothiolate-immobilized DNA monolayers as a function of time and temperature (25 to 90 °C). These results provide guidance on suitability of both chemistries for applications that require elevated temperatures. Examples of demonstrative applications are considered next, including measurement of surface melting transitions under reversible conditions for analysis of hybridization thermodynamics, and examination of the temperature dependence of kinetics of competitive surface hybridization when fully-matched and singly-mismatched targets compete for binding to a probe layer. Competitive surface hybridization equilibria are compared to predictions from individual sequence thermodynamics, measured with noncompetitive assays.

2. Materials and Methods

2.1. Materials

Poly(mercaptopropyl)methylsiloxane (PMPMS, degree of polymerization ~ 40, purity 95 %) was from Gelest, Inc. 1,11-bis-maleimidotriethyleneglycol (BM(PEG)₃) was from Pierce Biotechnology. Other chemicals were from Sigma-Aldrich or Fluka, reagent grade, and used as received. All aqueous solutions were prepared starting from deionized water (18.2 MΩ cm). Experiments used a 1 mol L⁻¹ (by phosphate groups) pH 7.2 sodium phosphate buffer, referred to as “SPB” buffer. DNA oligodeoxyribonucleotides were from Integrated DNA Technologies, and were purified by reverse phase high-performance liquid chromatography by the provider. The sequences and their modifications are listed in Table 1. The 20mer 5′ disulfide-modified probe sequences contained either a 3′ primary amine for labeling with a ferrocene tag (P-NH₂), or no 3′ modification (P). 20mer complementary full match target oligonucleotides were received with either a 3′ primary amine (FM-NH₂) or a 3′ disulfide (FM-S₂) for modification with amine- or thiol-reactive ferrocene tags, respectively. 20mer sequences with a single mismatch were obtained with and without a 3′ amine for ferrocene labeling (MM, MM-NH₂).

Procedures for labeling of amine-terminated oligonucleotides with *N*-hydroxysuccinimide ester of *N*-ferrocenylformylglycine (FcFG-NHS ester), and of disulfide-bearing oligonucleotides with *N*-ferrocenylacrylamide (FA0), were previously reported.³⁸ For the oligonucleotides in Table 1 these labeled products will be denoted as P-FcFG, FM-FcFG, FM-FA0, and MM-FcFG.

2.2. Preparation of PMPMS Films

1.6 mm diameter, polycrystalline gold disk working electrodes (Bioanalytical Systems) were mechanically polished with 1- μ m diamond slurry, rinsed with copious amounts of methanol followed by deionized water, and immediately electrochemically polished by cycling 30 times between -0.20 V and 1.75 V in 0.5 mol L⁻¹ H₂SO₄ at a scan rate of 100 mV s⁻¹. All potentials are quoted relative to an Ag/AgCl/ 3 mol L⁻¹ NaCl reference electrode. Following electrochemical polishing, cleaned electrodes were washed with deionized water and, without drying, the roughness factor r (r = actual area/geometric area) was determined from the double-layer capacitance as reported previously.^{39–40} Values of r ranged from 1.65 to 1.45 with an average of 1.55 . After roughness measurement, the electrodes were washed with deionized water, completely dried under a nitrogen stream, rinsed with anhydrous toluene solvent and transferred, while still wet, into 10 mmol L⁻¹ solutions (by monomer residue) of PMPMS in toluene for 1 h. After chemisorption of PMPMS, the modified electrodes were rinsed extensively with toluene and dried under a compressed nitrogen stream. The PMPMS-coated electrode surfaces were either characterized directly or modified further by reaction with BM(PEG)₃ (see below).

Gold coated slides (EMF Corp.) were used for ellipsometric measurements. The slides were cleaned by immersion in hot (~ 100 °C) “piranha” solution of 70/30 mixture of concentrated H₂SO₄ and 30% aqueous solution of H₂O₂ for 20 min, rinsed thoroughly with deionized water, and immediately electrochemically polished as described above. *Caution: piranha solution is highly corrosive and oxidizing and should never be stored in tightly capped containers due to gas evolution.* After washing with deionized water, roughness measurements were performed to determine r ; r was found to range from 1.1 to 1.25 with an average of 1.17 . Subsequent deposition of PMPMS was identical to that for disk electrodes.

2.3. Preparation of DNA Probe Monolayers

“C6-series” samples were based on chemistry introduced by Herne and Tarlov in 1997.⁴¹ In this approach, DNA probes are attached to gold through a terminal thiol, followed by passivation of the gold surface with 6-mercapto-1-hexanol (MCH). Gold disk electrodes were mechanically and electrochemically polished and characterized as described above, washed with deionized water and immediately immersed, while still wet, into 1 μ mol L⁻¹ solutions of P-FcFG in 1 mol L⁻¹ MgCl₂ for 30 min. After the probe immobilization step, electrodes were rinsed in deionized water and placed into 1 mmol L⁻¹ MCH in deionized water for 90 min to block the surface, then rinsed again with deionized water and SPB prior to use.

“AF-series” samples were prepared using PMPMS-coated disk electrodes. PMPMS electrodes were first immersed in 5 mmol L⁻¹ BM(PEG)₃ in dichloromethane (DCM) under stirring for 5 h, followed by thorough rinsing with DCM and drying under compressed nitrogen. Some of the BM(PEG)₃ is expected to form bridges between PMPMS thiols to crosslink the layer, and some BM(PEG)₃ is expected to undergo monodentate attachment to leave its second maleimide available for probe immobilization. The resultant crosslinked and maleimide-activated supports were immediately placed in 1 μ mol L⁻¹ solutions of thiol-terminated P-FcFG or thiol-terminated P in SPB at room temperature overnight. Thiol deprotection of the probes was performed with dithiothreitol (DTT) using 10 mmol L⁻¹

DTT in 10 mmol L⁻¹ TRIS, 1 mmol L⁻¹ EDTA, pH 8.0, for 2.5 h, followed by NAP-10 (GE Healthcare) and reverse-phase HPLC (Beckman Coulter Gold[®] 125; Clarity 3 μ m Oligo-RP column from Phenomenex[®]) purification. After probe immobilization, electrodes were rinsed with deionized water and SPB prior to use. Figure 1 illustrates the expected architectures for C6 and AF series samples.

For comparison, probe films were also prepared on uncrosslinked PMPMS anchor layers. For these experiments, 5 μ mol L⁻¹ solution of DNA-SH was reacted with a 100-fold excess of BM(PEG)₃ in SPB for 2 h while stirring. Maleimide-terminated DNA probes were recovered by passage through two separate NAP-10 columns, and the resultant $\sim$ 1 μ mol L⁻¹ probe solution in SPB was immediately contacted with a freshly-prepared, uncrosslinked PMPMS support. Immobilization was overnight at room temperature, followed by water and SPB rinses before use.

2.4. Electrochemical Setup

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out on a CHI 660C electrochemical workstation (CH Instruments Inc.). CV experiments used a water-jacketed cell connected to a circulating hot water bath for temperature control with an accuracy of ± 1 °C. The three-electrode cell configuration consisted of the 1.6 mm gold disk working electrode, modified as described above, a platinum wire counter electrode, and an Ag/AgCl/3 mol L⁻¹ NaCl reference electrode (Bioanalytical Systems; 0.209 V vs NHE at 25 °C). The reference electrode was isolated from the cell by a salt-bridge – a glass tube with a porous Vycor tip and filled with the same SPB electrolyte as the cell. EIS measurements were performed in the same three-electrode setup, under nonfaradaic conditions, and at room temperature ($\sim$ 22 °C). In all cases the SPB electrolyte was deoxygenated by nitrogen bubbling for 20 min, with nitrogen blanketing maintained throughout experiments. These precautions kept buffer O₂ concentrations to around 10 μ mol L⁻¹.

2.5. Analysis of Electrochemical Data

Strand coverages S were calculated from CV scans collected between 0 and 0.7 V at a 20 V s⁻¹ scan rate. S was obtained from $S = Q/(eA)$ where Q is the charge to switch the oxidation state of the ferrocene tags, e is the elementary charge, and A is the electrode area equal to the geometric area times the roughness factor. The division by $1e$ recognizes that ferrocenes undergo a one electron transfer process. Q was determined by fitting of tag peaks in CV traces, using a previously described algorithm.⁴²

The thickness of PMPMS films on gold disk electrodes was estimated from the differential capacitance C_d measured using EIS over the frequency range 10 Hz to 100 kHz, AC modulation of 5 mV rms, DC bias of 0 V, and under SPB electrolyte. C_d was obtained by modeling the data using an equivalent circuit of a solution resistance R_s in series with a constant phase element (CPE) to represent the charging response of the electrode. A CPE was used instead of a pure capacitor to allow for nonideal charging of the interface due to imperfections caused by roughness or chemical heterogeneity.⁴³ The CPE impedance is $Z_{CPE} = 1/((j\omega)^n Q)$ where ω is the AC frequency, n and Q are parameters characterizing the CPE, and j is square root of -1 . If $n = 1$ the CPE reduces to an ideal capacitor with $Q = C_d$, if $n = 0$ the CPE becomes a resistor with resistance $1/Q$. For the series R_s -CPE combination C_d follows from⁴⁴ $C_d = (R_s^{1-n} Q)^{1/n}/A$. R_s , Q , and n were determined by fitting raw EIS data using the ZSimpWin software (Princeton Applied Research). Values of n were 0.98 or higher, indicating close to ideal behavior. The EIS film thickness d was obtained from $d = \epsilon \epsilon_0 / C_d$, where $\epsilon = 6.0$ is the PMPMS dielectric constant⁴⁵ and ϵ_0 is the permittivity of free

space. This parallel plate relation assumes that C_d only reflects capacitance of the PMPMS layer.

2.6 Ellipsometry of PMPMS Films

EIS thickness measurements were confirmed with ellipsometry on a UVISSEL phase-modulated spectroscopic ellipsometer (HORIBA Jobin Yvon), using dried PMPMS films prepared on gold-coated slides (see section 2.2). Data were collected in the wavelength range 620 to 640 nm at 70° incidence. The ellipsometric angles were fit using a single layer model and manufacturer-supplied optical functions for gold, modeled as a semi-infinite support, and an extinction coefficient of zero and refractive index of 1.40 for the PMPMS layer, same as for poly(dimethyl siloxane).⁴⁶

2.7. Thermal Stability Studies

AF and C6 series P-FcFG films, prepared at initial probe coverages of $4 \times 10^{12} \text{ cm}^{-2}$, were subjected to stability tests consisting of three steps: (1) hybridization to FM-FA0 targets at 25 °C, (2) regeneration of unhybridized P-FcFG layers followed by heating to the desired test temperature and monitoring of the probe FcFG signal with time, and (3) once the FcFG signal decreased to about 50 %, cooling to 25 °C and re-hybridizing to FM-FA0 targets. Ideally, any decrease in FcFG electroactivity during step 2 would solely reflect loss due to probe desorption; however, at higher temperatures irreversible degradation of the ferrocene moieties via the oxidized ferricenium state also contributes.³⁸ Therefore, as a more robust measure of probe desorption, hybridization was performed under saturation conditions (see below) before (step 1) and after (step 3) the thermal treatment, with any decrease in hybridized amount attributed to probe loss.

Step 1 hybridizations were performed by immersion of freshly prepared probe films in 10 mL of $0.1 \mu\text{mol L}^{-1}$ FM-FA0 in SPB at 25 °C, while stirring, until the ferrocene FA0 peaks no longer increased (~ 40 min), thus signaling end of hybridization. The resultant probe and target coverages confirmed that extents of hybridization exceeded 90 %; i.e. that near saturation conditions were attained. The layers were dehybridized by a 15 s rinse with deionized water, the dehybridization was confirmed by absence of FA0 signal in CV, and step 2 was initiated by transfer of the dehybridized films into pre-heated SPB solutions at the desired test temperature. Transfer steps between solutions were performed while keeping the probe monolayer protected under a pendant droplet of the most recent solution. During step 2, CV scans were performed at designated times until the P-FcFG peak area decreased by approximately 50% from its starting value. Once this point was reached, monolayers were cooled off under a nitrogen stream while still wet, and then immersed in 10 mL of freshly prepared $0.1 \mu\text{mol L}^{-1}$ FM-FA0 in SPB at 25 °C to perform step 3 hybridization.

For C6 samples, because loss of MCH during step 2 could affect subsequent hybridization yields,⁴⁷ prior to initiating the step 3 hybridization it was necessary to refresh the MCH passivation by immersion in 1 mmol L^{-1} MCH in deionized water for 20 min.

2.8. Measurement and Analysis of Surface Melting Transitions

Unlabeled P probe (Table 1) monolayers, prepared according to the AF-protocol at coverages of $\sim 4 \times 10^{12} \text{ cm}^{-2}$, were placed under $0.05 \mu\text{mol L}^{-1}$ solutions of FcFG-labeled target in SPB. The temperature T was stepped manually from one value to the next, with a 10 min wait once each new value was reached, for an effective scan rate of about $0.3 \text{ }^\circ\text{C min}^{-1}$. Measurements consisted of a cooling scan from 70 to 25 °C, followed by dehybridization at 25 °C by rinsing with deionized water for 15 s, rehybridization at 25 °C for 2 h under the same target solution, and then a heating scan back to 70 °C under the target

solution. Superposition of cooling and heating scans was used to confirm reversible operation.

Thermodynamic analysis was performed on both cooling and heating scans. The equilibrium constant is $K \equiv S_D / [(S_0 - S_D)C_T] = \exp(-\Delta G^\circ / RT)$, where S_D is the duplex coverage, S_0 is the total probe coverage including both hybridized and unhybridized probes, C_T is the solution target concentration, and ΔG° is the standard free energy of surface hybridization. Substituting $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, and taking C_T as constant due to a large excess of targets, leads to

$$S_D = \frac{S_0 C_T \exp[-(\Delta H^\circ - T\Delta S^\circ) / RT]}{1 + C_T \exp[-(\Delta H^\circ - T\Delta S^\circ) / RT]} \quad (1)$$

Equation 1 was fit to melting transition data, in the form of experimental S_D as a function of temperature, with S_0 , ΔH° and ΔS° as fit parameters. The enthalpy ΔH° and entropy ΔS° of hybridization were assumed independent of temperature, following the conventional approximation of no change in heat capacity due to hybridization.^{48–49} Baseline corrections were not applied.

2.9. Measurement and Analysis of Melting Transitions in Solution

Solution melting curves were measured using oligonucleotides of the same sequence and modification, but without conjugated ferrocene labels, as for surface melting transitions. Probe and target strands were introduced into stoppered quartz cuvettes (Starna Cells) at a total strand concentration of $1 \mu\text{mol L}^{-1}$ (1:1 stoichiometry) in SPB. Duplex melting was monitored from DNA hyperchromicity changes at 260 nm, using a $0.2 \text{ }^\circ\text{C min}^{-1}$ ramp rate, on a Cary 50 spectrometer with a single-cuvette Peltier stage (Quantum Northwest). Two complete heat-cool cycles were performed to assure reversibility. Solution melting curves were fit to extract ΔH° and ΔS° as previously described,⁵⁰ using a $\pm 10 \text{ }^\circ\text{C}$ temperature range around the transition midpoint. Linear baselines were assumed at both high and low temperatures, and the temperature dependence of ΔH° and ΔS° was neglected.

2.10. Competitive Hybridization Measurements

Unlabeled P monolayers were prepared following the AF-series protocol. The films were immersed, while stirring, in deoxygenated, pre-heated SPB solutions containing $0.05 \mu\text{mol L}^{-1}$ FM-FcFG and $0.5 \mu\text{mol L}^{-1}$ unlabeled MM strands (Table 1), for a tenfold excess of the mismatch. Competitive hybridizations were performed at temperatures from 25 to $65 \text{ }^\circ\text{C}$, with CV scans taken every 2 min to monitor progress of hybridization. For comparison, noncompetitive hybridizations with FM-FcFG targets ($0.05 \mu\text{mol L}^{-1}$ in SPB) were performed in the temperature range 25 to $45 \text{ }^\circ\text{C}$.

Results of competitive assays were compared to theoretical predictions assuming that both the FM and MM hybridizations reached equilibrium. In that case,

$$K_{FM} = \frac{S_{D,FM}}{(S_0 - S_{D,FM} - S_{D,MM})C_{FM}} \text{ and } K_{MM} = \frac{S_{D,MM}}{(S_0 - S_{D,FM} - S_{D,MM})C_{MM}} \quad (2)$$

where K_i , $S_{D,i}$ and C_i are the surface equilibrium constant, surface duplex coverage, and solution concentration of species i . Equations 2 can be combined and rearranged to yield an expression for the equilibrium fraction f_{FM} of probes that hybridize to fully matched targets^{51–52}

$$f_{FM} = \frac{S_{D,FM}}{S_0} = \frac{1}{1 + 1/K_{FM} C_{FM} + \frac{C_{MM} K_{MM}}{C_{FM} K_{FM}}} \quad (3)$$

The ratio $K_{MM}/K_{FM} = \exp[-(\Delta H_{MM}^o - T\Delta S_{MM}^o)/RT] / \exp[-(\Delta H_{FM}^o - T\Delta S_{FM}^o)/RT] = \exp[-(\Delta\Delta H_{MM}^o - T\Delta\Delta S_{MM}^o)/RT]$, where $\Delta\Delta H_{MM}^o = \Delta H_{MM}^o - \Delta H_{FM}^o$ and $\Delta\Delta S_{MM}^o = \Delta S_{MM}^o - \Delta S_{FM}^o$ are the enthalpic and entropic costs of the mismatch. Combination with equation 3 yields

$$f_{FM} = \frac{1}{1 + 1/K_{FM} C_{FM} + \frac{C_{MM}}{C_{FM}} \exp[-(\Delta\Delta H_{MM}^o - T\Delta\Delta S_{MM}^o)/RT]} \quad (4)$$

Alternately, a kinetic model of competitive assays can be formulated (e.g. see ref. 53):

$$\frac{df_{FM}}{dt} = k_{f,FM} C_{FM} (1 - f_{FM} - f_{MM}) - k_{r,FM} f_{FM} \quad (5)$$

$$\frac{df_{MM}}{dt} = k_{f,MM} C_{MM} (1 - f_{FM} - f_{MM}) - k_{r,FM} f_{MM} \quad (6)$$

where $k_{f,i}$ and $k_{r,i} = k_{f,i}/K_i$ are the forward and reverse rate constants for surface hybridization of species i . Equations 5 and 6 neglect limitations due to mass transport, assume the rate constants to be independent of the extent of hybridization, and neglect contributions from strand displacement pathways in which invading targets displace previously bound strands from a probe.⁵⁴ The last assumption improves as temperature increases toward the duplex melting transition where dissociation of bound targets, represented by the reverse reactions in equations 5 and 6, dominates over strand displacement.⁵⁴

Results and Discussion

3.1. Thermostability of Probe Monolayers

Preservation of coverage and hybridization activity of probe monolayers was compared for the AF and C6 series samples as a function of temperature and time. The PMPMS films used for immobilization had a thickness $d = 2.6 \pm 0.1$ nm (3 samples) as revealed by both EIS and ellipsometric measurements. The close agreement between EIS and ellipsometry implies that double layer capacitive contributions to the EIS measurements were insignificant at the high ionic strengths used, so that the overall capacitance was dominated by that of the PMPMS dielectric. Based on earlier studies,⁴⁵ PMPMS films with a 2.6 nm thickness have about two thirds of their thiol groups (~ 7 groups nm⁻²) available for subsequent modification, with the other third forming thiolate bonds with the support.

The excess PMPMS thiols were extensively crosslinked with the homobifunctional crosslinker BM(PEG)₃. Reaction of BM(PEG)₃ through both its maleimides crosslinks PMPMS chains to one another, enhancing stability of the anchor film. For linkers that reacted through just one end, the second maleimide was available for attachment of DNA probes via terminal thiols. Variation of the BM(PEG)₃ crosslinking time, followed by probe immobilization, yielded probe coverages of 2.3×10^{12} cm⁻² on a PMPMS film crosslinked for 1 h, and 3.9×10^{12} and 4.0×10^{12} cm⁻² for films crosslinked for 3 and 5 h. These results suggest that 5 h was sufficient for BM(PEG)₃ to exhaust the supply of available PMPMS

thiols. Therefore, all subsequent studies used crosslinking times of 5 h and $\sim 4 \times 10^{12} \text{ cm}^{-2}$ probe coverages. The C6 series protocol in section 2.3 was chosen to produce comparable coverage films.

Figure 2 compares stability of the AF and C6 samples. The curves show decay of the probe FcFG signal during storage in SPB buffer at the indicated temperatures, corresponding to step 2 of the thermostability tests described in section 2.7. For comparison, at the end of each trace is a number that shows the percentage of retained hybridization activity after the heat treatment, given by the percent ratio of hybridized target post-treatment (step 3; section 2.7) to that observed pre-treatment (step 1; section 2.7). Hybridization was performed under saturating conditions; therefore, the percentage of retained activity directly measures how many active probes remained on the solid support. In contrast, the curves of FcFG activity tend to overestimate probe loss because they also reflect irreversible degradation of FcFG moieties, in addition to any desorption of probe strands. Retained hybridization percentage values are not shown for C6 samples at 80 and 90 °C because extensive degradation within minutes precluded the further use of these samples. The retained hybridization percentage for the AF-series at 25 °C refers to 60 h of storage under SPB – the time at which the FcFG signal decayed to 50 %.

Figure 2 shows that C6 samples (dashed lines) were less stable than AF samples (solid lines) at all temperatures. Moreover, for C6 samples, the decrease in probe FcFG signal with time correlated well with probe loss determined from retained hybridization capacity. For example, after 1.8 h at 65 °C the probe coverage decayed to 59 % of its starting value as estimated from FcFG activity, and to 67% as estimated from remnant hybridization capacity. The agreement between decrease in FcFG activity and hybridization capacity indicates that the FcFG signal, for C6 samples, predominantly tracks probe desorption rather than tag degradation. In this case the FcFG activity curves provide a reasonable approximation to layer stability and, based on a 20 % loss criterion, indicate that C6 monolayers are suitable for service for 2 to 3 hours at sustained temperatures of 45 °C and up to 10 h at 25 °C.

In contrast, for AF samples, decrease in FcFG activity reflects mostly FcFG degradation. For example, at the highest temperature of 90 °C, after 3 h AF samples still retained 90 % of active probes based on remnant hybridization capacity even though the FcFG signal decreased to just 49 % of its starting value. The excellent retention of hybridization activity for AF series samples is a first indication of their superior stability; this stability will be further highlighted below in the context of surface melting transition measurements.

The results in Figure 2 compare favorably to other efforts to realize thermostable probe layers. Recently, using a trithiol developed by Li *et al.*,²¹ Phares *et al.* showed that trithiol-anchoring restricted probe loss to just 20 % after nine simulated PCR cycles that cumulatively included 4 min at 95 °C, 8 min at 75 °C, and 4.5 min at 55 °C, as compared to 55 % loss observed with the conventional C6 monothiol immobilization after the same treatment.²² More recently, Civit *et al.* found that probe films immobilized through divalent diazonium attachment to gold exhibited a 7 % loss in hybridization, compared to 45 % for dithiol attachment, when such films were heated for 5 min at 15 degree intervals from 20 to 95 °C.¹⁹ Compared to these results, AFs based on PMPMS provide outstanding retention of hybridization activity when exposed to comparably harsh conditions over significantly longer times. The systematic data of the present study are also consistent with prior observations; e.g. using XPS measurements Johnson *et al.* reported that uncrosslinked PMPMS AFs, reacted with maleimide-terminated probes, retained about 90 % of probe DNA after 1 h at 95 °C,²⁰ while Lai *et al.* found a 40 % probe loss from a C6 sample after 7 days under ambient conditions.²³

3.2. Surface Melting Transitions

Melting transitions in solution are extensively used to investigate hybridization thermodynamics as well as interactions between nucleic acids and small ligands.^{55–56} Adaptation of melting transition measurements to a surface format demands thermostable probe films, whose organization and hybridization activity are not perturbed by measurements at elevated temperatures. Ideally, sufficient stability is available to allow confirmation of reversible operation from superposition of cooling and heating scans, so that each point on the melting transition can be retraced via two independent paths.

Figure 3A plots surface melting transitions for a probe layer formed on BM(PEG)₃-crosslinked PMPMS anchor films. Melting curves were measured while the film was kept under a 0.05 $\mu\text{mol L}^{-1}$ solution of FM-FcFG target in SPB. Two cycles of cooling and heating were performed in which temperature was stepped manually from one value to the next, with a 10 min wait once each new value was reached, for an effective scan rate of about 0.3 $^{\circ}\text{C min}^{-1}$. Following the first cooling scan the film was dehybridized, then rehybridized for 2 h at 25 $^{\circ}\text{C}$ to the same target solution before executing the heating scan. After completing a first such cool/heat cycle the probe layer was switched into a fresh target solution for the second cool/heat cycle. The melting curves show that the transitions were reversible, with only slight vertical offsets between heating and cooling scans attributed, at least in part, to degradation of target FcFG tags.

Compared to other immobilization methods,^{12–13, 17, 57–58} Figure 3A demonstrates that PMPMS anchor films can sustain sub 1 $^{\circ}\text{C min}^{-1}$ scan rates to provide near reversible operation, and to do so at sufficiently high temperatures to address the common scenario of 20mer or longer sequences with melting temperatures in excess of 50 $^{\circ}\text{C}$. In addition, the transitions retain a fairly sharp (~ 20 $^{\circ}\text{C}$ wide) character comparable to that of molecularly well-ordered C6-type DNA monolayers.^{12, 17, 58}

Interestingly, reversible behavior was lost when the same experiments were repeated with probes immobilized to uncrosslinked PMPMS films, Figure 3B. In the studies on uncrosslinked films, maleimide-terminated probes were attached directly to PMPMS thiols as described in section 2.3. Compared to Figure 3A, Figure 3B shows a gradual loss in hybridization activity, confirming that crosslinking of PMPMS with BM(PEG)₃ was essential to stabilization. The question remains whether loss in hybridization activity for uncrosslinked films was due to probe desorption or some other effect. To better understand this issue, a layer of unhybridized P-FcFG probes on uncrosslinked PMPMS films was tracked with CV at 65 $^{\circ}\text{C}$ for 3 h. After this time, 87 % of the FcFG signal remained. This can be compared to 89 % observed after the same treatment but when probes were instead tethered to crosslinked PMPMS films (Figure 2). The observation of similar decreases in probe signal whether or not the PMPMS was crosslinked indicates that crosslinking did not strongly impact stability of probe immobilization. Instead, the loss in hybridization activity on uncrosslinked PMPMS suggests that these films rearranged their organization in a way that penalized hybridization; for example, wrinkling of the films could have brought a fraction of probes into closer contact, thus suppressing the probes' ability to bind target strands. Since bulk PMPMS is liquid under the experimental conditions, responsiveness of PMPMS films to temperature changes would not be unexpected.

The notion that PMPMS layers can adjust their organization is further reinforced by the raw CV data presented in Figure 3C, illustrated for crosslinked layers. Even with crosslinking, as the temperature rose significant changes were observed in the baseline charging current which decreased from 25 to 50 $^{\circ}\text{C}$, followed by stabilization above 50 $^{\circ}\text{C}$. The stabilization occurred before the targets were fully melted off, suggesting it is not the melting process that is responsible. The changing baselines, which reverted with temperature, must therefore

reflect some temperature-dependent structural property of PMPMS. One possibility is that temperature changes altered the PMPMS/buffer interfacial tension, leading to changes in the roughness and thus the capacitance of this interface, as reflected in the baseline charging currents.

To illustrate comparison of mismatch penalties at surfaces with mismatch penalties in solution, surface melting transitions were measured for fully matched (FM) and mismatched (MM) targets hybridizing to unlabeled P probe monolayers. For these experiments, FM-FcFG and MM-FcFG targets were dissolved in SPB at two concentrations: 0.05 and 0.55 $\mu\text{mol L}^{-1}$. Fits were performed to cooling and, separately, heating half-cycles according to equation 1, with common values of ΔH° and ΔS° fit to both target concentrations. The surface melting data and optimized fits are plotted in Figures 4A and 4B for the FM and MM targets, respectively. For comparison, enthalpies and entropies of hybridization in solution were derived from solution melt curves, Figure 4C. Table 2 summarizes all of the derived parameters, including mismatch penalties $\Delta\Delta H^{\circ} = \Delta H^{\circ}_{\text{MM}} - \Delta H^{\circ}_{\text{FM}}$, $\Delta\Delta S^{\circ} = \Delta S^{\circ}_{\text{MM}} - \Delta S^{\circ}_{\text{FM}}$, and $\Delta\Delta G^{\circ} = \Delta G^{\circ}_{\text{MM}} - \Delta G^{\circ}_{\text{FM}}$. UNAFOLD⁵⁹ predictions for solution hybridization are shown in the bottom row of Table 2 for conditions most closely matched to those used experimentally.

In agreement with other reports,^{5, 13, 60–64} comparison of surface and solution ΔG° values in Table 2 shows that surface hybridization is significantly less favorable than solution hybridization, both for P/FM and P/MM duplexes. This difference may reflect the need to break up probe-probe associations or other interactions when targets hybridize to a probe layer,⁶⁰ noting that the ΔG° as defined by equation 1 will also include any costs of partitioning of targets into the layer from solution. The free energy cost of the mismatch at the surface, $\Delta\Delta G^{\circ}_{\text{MM}} = \Delta G^{\circ}_{\text{MM}} - \Delta G^{\circ}_{\text{FM}} = 1.6 \text{ kcal mol}^{-1}$, is found to be comparable to that in solution, $1.8 \text{ kcal mol}^{-1}$ (Table 2, last column). On the other hand, the enthalpic and entropic penalties $\Delta\Delta H^{\circ}_{\text{MM}}$ and $\Delta\Delta S^{\circ}_{\text{MM}}$ associated with the mismatch appear to double at the surface, with the differences compensating in calculation of $\Delta\Delta G^{\circ}_{\text{MM}}$. This ability to compensate, however, was also seen to lead to significant enthalpy-entropy correlation during fitting;⁶⁵ therefore, the most reliable metric for comparison is the free energy, with enthalpy and entropy more appropriately viewed as formal fitted values rather than accurate physical quantities.

3.3. Temperature Dependence of Competitive Hybridization

The second demonstration of an application based on PMPMS immobilization illustrates the impact of temperature on competitive surface hybridization. In these experiments two targets, a fully matched (FM) and a mismatched (MM) sequence (Table 1), sought to simultaneously hybridize to the probes.

Results from a series of competitive hybridization experiments are shown in Figure 5A, which plots the percent f_{FM} of probes hybridized to the fully matched target FM-FcFG from a solution containing a ten-fold excess of unlabeled MM mismatch sequences; i.e. 0.5 $\mu\text{mol L}^{-1}$ MM to 0.05 $\mu\text{mol L}^{-1}$ FM-FcFG. These studies were performed as a function of temperature T , with measurements taken at various times after initiation of hybridization. As seen in Figure 5A, the f_{FM} versus T trend was found to be non-monotonic with a maximum at intermediate temperatures.

The experimental dependence of f_{FM} on T can be considered starting from the high temperature limit where, as temperature decreases, f_{FM} initially rose due to stabilization of probe-target duplexes. From 65 to about 55 $^{\circ}\text{C}$ the f_{FM} values for the various times converged, indicating achievement of equilibrium. This temperature range falls well within the surface melting transitions in Figure 4 where dehybridization rates are significant, thus

ensuring turnover of targets on the probes needed for equilibration of the hybridized and solution target populations. Further decreases in temperature, however, caused f_{FM} for the different times to diverge as well as decrease, falling to just 8 % at 25 °C. These changes indicate that equilibrium was no longer reached; indeed, as shown in Figure 5B by the calculated equilibrium predictions for three different sets of thermodynamic inputs, for any reasonable combination of inputs equilibrium would require f_{FM} to continue to increase, not decrease, as temperature was lowered.

Since the experimentally observed suppression of f_{FM} at low T was not observed under noncompetitive conditions (e.g. a 1 h noncompetitive hybridization at 25 °C produced $f_{\text{FM}} = 0.9$), suppression for the competitive scenario is attributed to occupation, and thus blockage, of probes by cross-hybridized MM sequences. This explanation agrees with the calculated kinetic trend for competitive hybridization of FM and MM targets, corresponding to the black dashed line in Figure 5B. As observed previously,^{53, 67–70} when a mismatched but hybridizable sequence is present in excess most probes will initially bind the mismatch. The data of Figure 5 illustrate that transitioning from this initial nonequilibrium state to equilibrium is dramatically dependent on temperature, becoming prolonged at lower temperatures. The strict pinning with time of f_{FM} at 25 °C is attributed to kinetic freezing due to negligible target turnover at this low temperature, as expected based on 25 °C falling below the surface melting transitions (cf. Figures 4A and 4B). Related temperature effects were recently also found important in temperature-based annealing to improve detection of point mutations.⁷¹

Somewhat surprisingly, the equilibrium predictions in Figure 5B based on enthalpies and entropies for surface hybridization (red curve) did not agree with the experimental data any better than predictions based on solution thermodynamics (green curve). However, better agreement was realized if the surface values were slightly adjusted, for example, to the following settings (blue curve): $\Delta H_{\text{FM}}^{\text{P}} = -64,000$ instead of $-63,000$ cal mol⁻¹, $\Delta S_{\text{FM}}^{\text{P}} = -159$ instead of -160 cal mol⁻¹ K⁻¹, $\Delta \Delta H_{\text{MM}}^{\text{P}} = 15,600$ cal mol⁻¹ (unchanged), and $\Delta \Delta S_{\text{MM}}^{\text{P}} = 43$ instead of 45 cal mol⁻¹ K⁻¹. The “adjusted” calculations make the important point that uncertainties of a few percent in thermodynamic inputs can significantly impact the predicted trend of f_{FM} with temperature. The current modeling of surface melting curves, from which the surface thermodynamic inputs were derived, may not be sufficiently refined to distinguish such small differences. For one, above-mentioned fitting correlations between enthalpy and entropy, while compensated at the location of a melting transition, will lead to errors with extrapolation to other temperatures. Moreover, Figures 4A and 4B show that the calculated melting transitions are slightly, but reproducibly, sharper than the experimental ones, suggesting the model is missing a salient feature of the real system.⁷² Such sharper fits were also reported by Fiche *et al.*¹⁵ and Vainrub *et al.*^{16, 73} who noted that broadening of experimental surface melting transitions could arise from a dispersion in hybridization thermodynamics; e.g. due to local variations in probe coverage^{16, 73} or in number of probe nucleotides accessible for hybridization.¹⁵

While multiple explanations can be held responsible for broadening of surface melt curves, the notion that probes experience a range of local environments that impact hybridization is general, and consistent with the present data. Sloping baselines at low temperatures, as in Figures 4A and 4B, provide further evidence of variability in probe activity, indicating that certain probes could not hybridize until sufficiently low temperatures were reached. A conceptually straightforward, though challenging to implement, approach to eliminating such complications would be to design probe layers in which the environment surrounding each probe is identical.

4. Conclusions

DNA probe monolayers immobilized through thioether bonds to nanometer-thick anchor films of crosslinked poly(mercaptopropyl)methylsiloxane (PMPMS) on gold retained hybridization activity toward target strands for hours at temperatures of up to 90 °C. Crosslinking of the PMPMS was essential to robust preservation of hybridization activity; this was argued to reflect the need to secure the polymer against structural rearrangements. In comparison, probes attached solely through a single thiolate bond desorbed within minutes under comparably harsh conditions.

Stability of PMPMS-tethered probe films was exploited to (1) demonstrate measurement of surface melting transitions under reversible conditions and (2) examine effects of temperature on competitive surface hybridization of fully-matched (FM) and singly-mismatched (MM) targets. When kept under solutions of target molecules, PMPMS-immobilized probes exhibited well-defined surface melting transitions whose reversibility could be verified from superposition of heating and cooling half-cycles. Standard two-state models, adapted from solution methods, were used to derive enthalpies and entropies of surface hybridization for FM and MM probe-target pairs. Solution melting experiments were performed for comparison. These studies showed that while total free energies of surface hybridization were suppressed relative to those in solution, the mismatch free energy penalty at the surface and in solution were similar. Competitive surface hybridization of FM and MM targets exhibited pronounced dependence on temperature, ranging from kinetic freezing at initially-formed FM and MM coverages at room temperatures to facile equilibration at temperatures sufficiently high to fall within the FM and MM surface melting transitions. Modeling of competitive hybridization and melting curve equilibria at surfaces suggested that dispersity in probe states needs to be better understood, and controlled, to develop predictive capability that is comparable to existing models of solution hybridization.

Acknowledgments

This project was supported by Award No. R33HG003089 from the National Human Genome Research Institute, National Institutes of Health, and by Awards No. DMR 07-06170 and DGE 07-41714 from the National Science Foundation.

References

1. Jordan BR. Is there a niche for DNA microarrays in molecular diagnostics? *Expert Rev Mol Diag.* 2010; 10:875–882.
2. Li X, Quigg RJ, Zhou J, Gu W, Nagesh RP, Reed EF. Clinical utility of microarrays: Current status, existing challenges and future outlook. *Curr Genomics.* 2008; 9:466–474. [PubMed: 19506735]
3. Müller, H-J.; Röder, T. *Microarrays.* Elsevier Academic Press; Burlington, MA: 2006.
4. Langdon WB, Upton GJG, Harrison AP. Probes containing runs of guanines provide insights into the biophysics and bioinformatics of Affymetrix GeneChips. *Brief Bioinform.* 2009; 10:259–277. [PubMed: 19359259]
5. Levicky R, Horgan A. Physicochemical perspectives on DNA microarray and biosensor technologies. *Trends Biotechnol.* 2005; 23:143–149. [PubMed: 15734557]
6. Pozhitkov AE, Tautz D, Noble PA. Oligonucleotide microarrays: Widely applied-poorly understood. *Brief Funct Genomic Proteomic.* 2007; 6:141–148. [PubMed: 17644526]
7. Pozhitkov A, Noble PA, Domazet-Lozo T, Nolte AW, Sonnenberg R, Staehler P, Beier M, Tautz D. Tests of rRNA hybridization to microarrays suggest that hybridization characteristics of oligonucleotide probes for species discrimination cannot be predicted. *Nucl Acids Res.* 2006; 34:e66. [PubMed: 16707658]

8. Sykacek P, Kreil DP, Meadows LA, Auburn RP, Fischer B, Russell S, Micklem G. The impact of quantitative optimization of hybridization conditions on gene expression analysis. *BMC Bioinformatics*. 2011; 12:art. 73.
9. Wu P, Castner DG, Grainger DW. Diagnostic devices as biomaterials: A review of nucleic acid and protein microarray surface performance issues. *J Biomat Sci - Polym Ed*. 2008; 19:725–753.
10. Stimpson DI, Hoijer JV, Hsieh W, Jou C, Gordon J, Theriault T, Gamble R, Baldeschwieler JD. Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides. *Proc Natl Acad Sci U S A*. 1995; 92:6379–6383. [PubMed: 7603999]
11. Forman JE, Walton ID, Stern D, Rava RP, Trulson MO. Thermodynamics of duplex formation and mismatch discrimination on photolithographically synthesized oligonucleotide arrays. *ACS Sym Ser*. 1998; 682:206–228.
12. Peterlinz KA, Georgiadis RM, Herne TM, Tarlov MJ. Observation of hybridization and dehybridization of thiol-tethered DNA using two-color surface plasmon resonance spectroscopy. *J Am Chem Soc*. 1997; 119:3401–3402.
13. Xu J, Craig SL. Thermodynamics of DNA hybridization on gold nanoparticles. *J Am Chem Soc*. 2005; 127:13227–13231. [PubMed: 16173751]
14. Piunno PAE, Watterson JH, Kotoris CC, Krull UJ. Alteration of the selectivity of hybridization of immobilized oligonucleotide probes by co-immobilization with charged oligomers of ethylene glycol. *Anal Chim Acta*. 2005; 534:53–61.
15. Fiche JB, Buhot A, Calemczuk R, Livache T. Temperature effects on DNA chip experiments from surface plasmon resonance imaging: Isotherms and melting curves. *Biophys J*. 2007; 92:935–946. [PubMed: 17085497]
16. Vainrub A, Deluge N, Zhang X, Zhou X, Gao X. Predicting DNA duplex stability on oligonucleotide arrays. *Methods Mol Biol*. 2007; 382:393–403. [PubMed: 18220244]
17. Fuchs J, Fiche JB, Buhot A, Calemczuk R, Livache T. Salt concentration effects on equilibrium melting curves from DNA microarrays. *Biophys J*. 2010; 99:1886–1895. [PubMed: 20858434]
18. Bodrossy L, Sessitsch A. Oligonucleotide microarrays in microbial diagnostics. *Curr Opin Microbiol*. 2004; 7:245–254. [PubMed: 15196491]
19. Civit L, Frago A, O'Sullivan CK. Thermal stability of diazonium derived and thiol-derived layers on gold for application in genosensors. *Electrochem Commun*. 2010; 12:1045–1048.
20. Johnson PA, Levicky R. Polymercaptosiloxane anchor films for robust immobilization of biomolecules to gold supports. *Langmuir*. 2003; 19:10288–10294.
21. Li Z, Jin R, Mirkin CA, Letsinger RL. Multiple thiol-anchor capped DNA-gold nanoparticle conjugates. *Nucl Acids Res*. 2002; 30:1558–1562. [PubMed: 11917016]
22. Phares N, White RJ, Plaxco KW. Improving the stability and sensing of electrochemical biosensors by employing trithiol-anchoring groups in a six-carbon self-assembled monolayer. *Anal Chem*. 2009; 81:1095–1100. [PubMed: 19133790]
23. Lai RY, Seferos DS, Heeger AJ, Bazan GC, Plaxco KW. Comparison of the signaling and stability of electrochemical DNA sensors fabricated from 6- or 11-carbon self-assembled monolayers. *Langmuir*. 2006; 22:10796–10800. [PubMed: 17129062]
24. Johnson PA, Gaspar MA, Levicky R. Polymer-anchored DNA gene monolayers. *J Am Chem Soc*. 2004; 126:9910–9911. [PubMed: 15303851]
25. Dougan JA, Karlsson C, Smith WE, Graham D. Enhanced oligonucleotide-nanoparticle conjugate stability using thioctic acid modified oligonucleotides. *Nucl Acids Res*. 2007; 35:3668–3675. [PubMed: 17488844]
26. Sharma J, Chhabra R, Yan H, Liu Y. A facile *in situ* generation of dithiocarbamate ligands for stable gold nanoparticle-oligonucleotide conjugates. *Chem Commun*. 2008:2140–2142.
27. Sakata T, Maruyama S, Ueda A, Otsuka H, Miyahara Y. Stable immobilization of an oligonucleotide probe on a gold substrate using tripodal thiol derivatives. *Langmuir*. 2007; 23:2269–2272. [PubMed: 17269799]
28. Su XD, Wu YJ, Knoll W. Comparison of surface plasmon resonance spectroscopy and quartz crystal microbalance techniques for studying DNA assembly and hybridization. *Biosens Bioelectron*. 2005; 21:719–726. [PubMed: 16242610]

29. Nelson BP, Grimsrud TE, Liles MR, Goodman RM, Corn RM. Surface plasmon resonance imaging measurements of DNA and RNA hybridization adsorption onto DNA microarrays. *Anal Chem.* 2001; 73:1–7. [PubMed: 11195491]
30. Okahata Y, Kawase M, Niikura K, Ohtake F, Furusawa H, Ebara Y. Kinetic measurements of DNA hybridisation on an oligonucleotide-immobilized 27-MHz quartz crystal microbalance. *Anal Chem.* 1998; 70:1288–1296. [PubMed: 15779144]
31. Peterson AW, Heaton RJ, Georgiadis RM. The effect of surface probe density on DNA hybridization. *Nucl Acids Res.* 2001; 29:5163–5168. [PubMed: 11812850]
32. Ricci F, Lai RY, Heeger AJ, Plaxco KW, Sumner JJ. Effect of molecular crowding on the response of an electrochemical DNA sensor. *Langmuir.* 2007; 23:6827–6834. [PubMed: 17488132]
33. Wong ELS, Chow E, Gooding JJ. DNA recognition interfaces: The influence of interfacial design on the efficiency and kinetics of hybridization. *Langmuir.* 2005; 21:6957–6965. [PubMed: 16008409]
34. Gong P, Levicky R. DNA surface hybridization regimes. *Proc Natl Acad Sci U S A.* 2008; 105:5301–5306. [PubMed: 18381819]
35. Gong P, Lee CY, Gamble LJ, Castner DG, Grainger DW. Hybridization behavior of mixed DNA/alkylthiol monolayers on gold: Characterization by surface plasmon resonance and ³²P radiometric assay. *Anal Chem.* 2006; 78:3326–3334. [PubMed: 16689533]
36. Livache T, Roget A, Dejean E, Barthet C, Bidan G, Teoule R. Preparation of a DNA matrix via an electrochemically directed copolymerization of pyrrole and oligonucleotides bearing a pyrrole group. *Nucl Acids Res.* 1994; 22:2915–2921. [PubMed: 8065902]
37. Neumann T, Bonham AJ, Dame G, Berchtold B, Brandstetter T, Ruhe J. Temperature and time-resolved total internal reflectance fluorescence analysis of reusable DNA hydrogel chips. *Anal Chem.* 2010; 82:6124–6131. [PubMed: 20552989]
38. Ge D, Levicky R. A comparison of five bioconjugatable ferrocenes for labeling of biomolecules. *Chem Commun.* 2010; 46:7190–7192.
39. Oesch U, Janata J. Electrochemical study of gold electrodes with anodic oxide films - I. Formation and reduction behavior of anodic oxides on gold. *Electrochim Acta.* 1983; 28:1237–1246.
40. Shen G, Tercero N, Gaspar MA, Varughese B, Shepard K, Levicky R. Charging behavior of single-stranded DNA polyelectrolyte brushes. *J Am Chem Soc.* 2006; 128:8427–8433. [PubMed: 16802807]
41. Herne TM, Tarlov MJ. Characterization of DNA probes immobilized on gold surfaces. *J Am Chem Soc.* 1997; 119:8916–8920.
42. Tercero N, Wang K, Gong P, Levicky R. Morpholino monolayers: Preparation and label-free DNA analysis by surface hybridization. *J Am Chem Soc.* 2009; 131:4953–4961. [PubMed: 19296583]
43. Scheider W. Theory of the frequency dispersion of electrode polarization. Topology of networks with fractional power frequency dependence. *J Phys Chem.* 1975; 79:127–136.
44. Brug GJ, van den Eeden ALG, Sluyters-Rehbach M, Sluyters JH. The analysis of electrode impedances complicated by the presence of a constant phase element. *J Electroanal Chem.* 1984; 176:275–295.
45. Johnson PA, Levicky R. X-ray photoelectron spectroscopy and differential capacitance study of thiol-functional polysiloxane films on gold supports. *Langmuir.* 2004; 20:9621–9627. [PubMed: 15491195]
46. Tsao MW, Pfeifer KH, Rabolt JF, Castner DG, Haussling L, Ringsdorf H. Formation and characterization of self-assembled films of thiol-derivatized poly(dimethylsiloxane) on gold. *Macromolecules.* 1997; 30:5913–5919.
47. Levicky R, Herne TM, Tarlov MJ, Satija SK. Using self-assembly to control the structure of DNA monolayers on gold: A neutron reflectivity study. *J Am Chem Soc.* 1998; 120:9787–9792.
48. Breslauer KJ, Sturtevant JM, Tinoco IJ. Calorimetric and spectroscopic investigation of the helix-to-coil transition of a ribo-oligonucleotide: rA₇U₇. *J Mol Biol.* 1975; 99:549–556. [PubMed: 1214299]
49. Mikulecky PJ, Feig AL. Heat capacity changes associated with nucleic acid folding. *Biopolymers.* 2006; 82:38–58. [PubMed: 16429398]

50. Gong P, Wang K, Liu Y, Shepard K, Levicky R. Molecular mechanisms in morpholino-DNA surface hybridization. *J Am Chem Soc.* 2010; 132:9663–9671. [PubMed: 20572663]
51. Halperin A, Buhot A, Zhulina EB. Hybridization isotherms of DNA microarrays and the quantification of mutation studies. *Clin Chem.* 2004; 50:2254–2262. [PubMed: 15388635]
52. Halperin A, Buhot A, Zhulina EB. On the hybridization isotherms of DNA microarrays: The Langmuir model and its extensions. *J Phys: Condens Mat.* 2006; 18:S463–S490.
53. Bishop J, Blair S, Chagovetz AM. A competitive kinetic model of nucleic acid surface hybridization in the presence of point mutants. *Biophys J.* 2006; 90:831–840. [PubMed: 16284267]
54. Reynaldo LP, Vologodskii AV, Neri BP, Lyamichev VI. The kinetics of oligonucleotide replacements. *J Mol Biol.* 2000; 297:511–520. [PubMed: 10715217]
55. Breslauer KJ. Extracting thermodynamic data from equilibrium melting curves for oligonucleotide order-disorder transitions. *Methods Enzymol.* 1995; 259:221–242. [PubMed: 8538456]
56. Wilson WD, Tanious FA, Fernandez-Saiz M, Rigl CT. Evaluation of drug-nucleic acid interactions by thermal melting curves. *Methods Mol Biol.* 1994; 90:219–240. [PubMed: 9407538]
57. Biswal SL, Raorane D, Chaiken A, Birecki H, Majumdar A. Nanomechanical detection of DNA melting on microcantilever surfaces. *Anal Chem.* 2006; 78:7104–7109. [PubMed: 17037909]
58. Nasef H, Ozalp VC, Beni V, O'Sullivan CK. Melting temperature of surface-tethered DNA. *Anal Biochem.* 2010; 406:34–40. [PubMed: 20615383]
59. Markham, NR.; Zuker, M. *Bioinformatics, Volume II Structure, Functions and Applications.* Keith, JM., editor. Vol. 453. Humana Press; Totowa, NJ: 2008. p. 3-31.
60. Irving D, Gong P, Levicky R. DNA surface hybridization: Comparison of theory and experiment. *J Phys Chem B.* 2010; 114:7631–7640. [PubMed: 20469913]
61. Chen CL, Wang WJ, Ge J, Zhao XS. Kinetics and thermodynamics of DNA hybridization on gold nanoparticles. *Nucl Acids Res.* 2009; 37:3756–3765. [PubMed: 19380378]
62. Watterson JH, Piuino PAE, Wust CC, Krull UJ. Effects of oligonucleotide immobilization density on selectivity of quantitative transduction of hybridization of immobilized DNA. *Langmuir.* 2000; 16:4984–4992.
63. Carlon E, Heim T. Thermodynamics of RNA/DNA hybridization in high density oligonucleotide microarrays. *Physica A.* 2006; 362:433–449.
64. Li SZ, Pozhitkov A, Brouwer M. A competitive hybridization model predicts probe signal intensity on high density DNA microarrays. *Nucl Acids Res.* 2008; 36:6585–6591. [PubMed: 18931378]
65. For example, constraining the surface $\Delta\Delta H^\circ$ to its solution value ($8000 \text{ cal mol}^{-1}$) during fitting was compensated by an adjustment in $\Delta\Delta S^\circ$ to $22 \text{ cal mol}^{-1} \text{ K}^{-1}$, with only a 20 % increase in the least square error.
66. Liu Y, Irving D, Qiao W, Ge D, Levicky R. Kinetic mechanisms in morpholino-DNA surface hybridization. *J Am Chem Soc.* 2011; 133:11588–11596. [PubMed: 21699181]
67. Bhanot G, Louzoun Y, Zhu J, DeLisi C. The importance of thermodynamic equilibrium for high throughput gene expression arrays. *Biophys J.* 2003; 84:124–135. [PubMed: 12524270]
68. Zhang Y, Hammer DA, Graves DJ. Competitive hybridization kinetics reveals unexpected behavior patterns. *Biophys J.* 2005; 89:2950–2959. [PubMed: 16126833]
69. Horne MT, Fish DJ, Benight AS. Statistical thermodynamics and kinetics of DNA multiplex hybridization reactions. *Biophys J.* 2006; 91:4133–4153. [PubMed: 16963510]
70. Chechetkin VR. Two-compartment model for competitive hybridization on molecular biochips. *Physics Letters A.* 2007; 360:491–494.
71. Pingel J, Buhot A, Calemczuk R, Livache T. Temperature scans/cycles for the detection of low abundant DNA point mutations on microarrays. *Biosens Bioelectron.* 2012; 31:554–557. [PubMed: 22047974]
72. Modeling of heat capacity contributions within expected ranges of the ΔC_p for hybridization (e.g. see Mikulecky, Feig. *Biopolymers.* 2006; 82:38–58. [PubMed: 16429398]) was not able to account for the greater sharpness of calculated transitions.
73. Vainrub A, Pettitt BM. Accurate prediction of binding thermodynamics for DNA on surfaces. *J Phys Chem B.* 2011; 115:13300–13303. [PubMed: 21972932]

Figure 1.
 Expected organization of the AF- and C6-series samples.

Figure 2.

Decrease in electroactivity of probe FcFG tags with time as monitored in SPB buffer at different temperatures for AF (solid lines) and C6 (dashed lines) probe layers. For clarity, data at 80 and 90 °C are shown separately. Numbers at end of the curves indicate percentage of remnant hybridization capacity after the heat treatment relative to that before the heat treatment (cf. section 2.7). See text for discussion.

Figure 3. Surface melting transitions on (A) crosslinked and (B) uncrosslinked PMPMS anchor films. Temperature ramps were performed between 25 and 70 $^{\circ}\text{C}$ at $0.3 \text{ }^{\circ}\text{C min}^{-1}$ under $0.05 \text{ } \mu\text{mol L}^{-1}$ FM-FcFG target in SPB. (C) Select CV traces corresponding to the "cooling 1" scan in part (A). Probe coverage: $4 \times 10^{12} \text{ cm}^{-2}$.

Figure 4.

Surface melting transitions for hybridization of P probes with (A) FM-FcFG and (B) MM-FcFG targets, showing both cooling (—) and heating (---) ramps. Fits (—), shown for the cooling half-cycles only, were performed simultaneously to data from both the 0.05 and 0.55 $\mu\text{mol L}^{-1}$ target concentrations in SPB. Fit parameters in equation 1 included S_0 (independent for each concentration), ΔH^0 (shared), and ΔS^0 (shared). (C) Solution melting transitions for P/FM and P/MM duplexes, measured with UV absorbance at 1 $\mu\text{mol L}^{-1}$ total strand concentration in SPB. Points (●): data. Lines (—): fits.

Figure 5.

(A) Evolution with time and temperature of the percent fraction of probes hybridized to fully matched targets, f_{FM} , during competitive surface hybridization to a 1:10 (0.05:0.5 $\mu\text{mol L}^{-1}$) mixture of FM-FcFG to MM targets in SPB. (B) Comparison of experimental data, at 60 min, with kinetic and equilibrium calculations. Equilibrium calculations are based on equation 4 and surface (Table 2, top row), solution (Table 2, middle row), or adjusted ($\Delta H_{FM}^{\circ} = -64,000 \text{ cal mol}^{-1}$, $\Delta S_{FM}^{\circ} = -159 \text{ cal mol}^{-1} \text{ K}^{-1}$, $\Delta \Delta H_{MM}^{\circ} = 15,600 \text{ cal mol}^{-1}$, $\Delta \Delta S_{MM}^{\circ} = 43 \text{ cal mol}^{-1} \text{ K}^{-1}$) thermodynamic inputs. Kinetic predictions are for a hybridization time of 60 min and are based on equations 5 and 6, with the “adjusted” enthalpy and entropy used to derive equilibrium constants K for the FM and MM targets. Reverse rate constants were obtained using $k_r = k_f/K$, where $k_f = 2 \times 10^5 \text{ L mol}^{-1} \text{ s}^{-1}$ was taken as a typical value for both FM and MM hybridization based on review of reported surface rate constants.⁶⁶

Table 1Oligonucleotide Sequences.^a

Acronym	Sequence
P	5'-HO-(CH ₂) ₆ -SS-(CH ₂) ₆ -CAG GCA CAA ACA TGA ACC TC-3'
P-NH ₂	5'-HO-(CH ₂) ₆ -SS-(CH ₂) ₆ -CAG GCA CAA ACA TGA ACC TC-(CH ₂) ₆ -NH ₂ -3'
FM-NH ₂	5'-GAG GTT CAT GTT TGT GCC TG-(CH ₂) ₆ -NH ₂ -3'
FM-S ₂	5'-GAG GTT CAT GTT TGT GCC TG-(CH ₂) ₃ -SS-(CH ₂) ₃ -OH-3'
MM	5'-GAG GTT <u>CGT</u> GTT TGT GCC TG-3'
MM-NH ₂	5'-GAG GTT <u>CGT</u> GTT TGT GCC TG-(CH ₂) ₆ -NH ₂ -3'

^aP: probe sequence; FM: target sequence fully matched to P; MM: mismatched target sequence (underline indicates mismatch).

Table 2

Comparison of Surface and Solution Hybridization Thermodynamics^a

	fully matched target (FM)			mismatched target (MM)			difference (MM-FM)		
	ΔH°_{FM}	ΔS°_{FM}	ΔG°_{FM}	ΔH°_{MM}	ΔS°_{MM}	ΔG°_{MM}	ΔH°_{MM}	$\Delta \Delta S^{\circ}_{MM}$	$\Delta \Delta G^{\circ}_{MM}$
Surface	-63.0 ± 0.3^b	-160 ± 1^b	-13.4^d	-47.4 ± 0.6^b	-115 ± 2^b	-11.8^d	15.6	45	1.6 ^d
Solution	-161	-439	-24.9^d	-153	-419	-23.1^d	8	20	1.8 ^d
UNFD^c	-156	-424	-24.6^d	-147	-401	-22.7^d	9	23	1.9 ^d

^aEnthalpies and free energies are in kcal mol⁻¹, entropies are in cal mol⁻¹ K⁻¹.

^bSurface ΔH° and ΔS° are averages of values derived from cooling and heating half cycles.

^cUNAFOLD predictions are for 1 mol L⁻¹ Na⁺ concentration, the highest value allowed by the software. Experimental data were obtained in SPB, corresponding to a 1.55 mol L⁻¹ Na⁺ concentration.

^dEvaluated at 37 °C.