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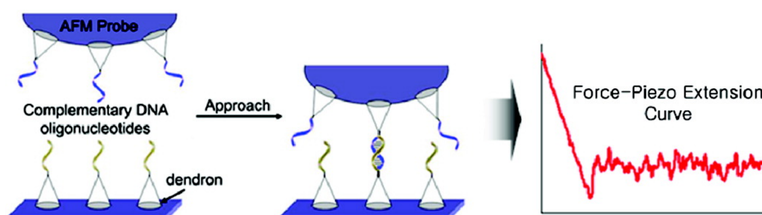

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Dendron Arrays for the Force-Based Detection of DNA Hybridization Events

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Abstract: Single-molecule force measurement methods have attracted increasing interest over recent years for the development of novel approaches for biomolecular screening. However, many of these developments are currently hindered by the available biomolecule surface attachment methods, in that it is still not trivial to create surfaces and devices with highly defined surface functionality and/or uniformity. Here we offer a new approach to address such issues based on the formation of dendron arrays. Through the measurement of forces between dendron surfaces functionalized with complementary DNA oligonucleotides, we observed several unique properties of the surfaces modified via this approach. The capability to record attractive or "jump-in" forces associated with molecular binding events is one of them. Additionally, these events occur in greater than 80% of measurements, and the forces are dependent on the number of complementary DNA bases of the associating strands while being insensitive to the measurement rate. Combined with a narrow distribution of both attractive forces and unbinding forces we suggest such functionalized surfaces offer a significant advance for fast and accurate force-based studies of oligonucleotide hybridization.

Introduction

Over the past decade an increasing number of studies have demonstrated the ability of ultrasensitive force measurement methods to study the unbinding kinetics of single molecular receptor/ligand pairs and to probe the mechanical properties of single biopolymeric molecules.[1−4] Such studies have provided much novel insight into the role of force in a range of biological processes, including in cell adhesion,[5−7] in protein unfolding,[8−10] and on the dissociation of DNA/RNA oligonucleotide duplexes.[11−13] As a consequence, researchers have begun to consider force as an important parameter to include in studies of biomolecular structure and function.

In parallel with these developments, a significant growth in the number of quantitative and comprehensive studies on the genome for drug discovery, as well as for disease diagnosis and prevention, has placed a strong demand for advanced biomolecular recognition probes with high sensitivity and excellent specificity.[14] Not surprisingly, much attention has focused on methods that are able to provide data on the behavior of single biological molecules,[15] including those that are based on force detection.[16,17] For nucleic acid based detection methods, interest in this latter area has arisen through a realization that knowledge of the mechanical properties of nucleic acids is of crucial importance for a deep understanding of numerous important biological processes, such as DNA transcription and replication, and also in gene expression and regulation.

The force-extension and force-induced melting properties of nucleic acids, and also their complexes with other molecules, have been investigated using several force measurement tech-

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niques, including optical tweezers and atomic force microscopy (AFM).[2−4,11−13,18−21] Particularly relevant for the screening of nucleic acid hybridization events, and hence also for single base mismatch/polymorphism detection technologies, are studies which have revealed that the unbinding forces recorded between oligonucleotide sequences are highly dependent on the number of complementary bases.[11−13] Indeed, by exploiting these predictable and essentially “programmable” properties, researchers have recently developed force-based DNA[16] and protein chips[17] in which, via a differential parallel array format, the unbinding forces of individual target biomolecular complexes (e.g., receptor−ligand or DNA duplex) are compared to that required to unzip/unbind reference DNA duplexes. This format was developed to generate a force-based method that was compatible with standard chip-based assays but, more importantly, to remove most of the experimental uncertainties implicit in these measurements associated with external factors (e.g., drift, temperatures, etc.) and instrumentation, factors which have been significant bottle-necks to the wide acceptance of force-based methods for screening applications.

While such studies are extremely elegant and remove many of the unwanted experimental variables associated with single-molecule force measurements, they still ultimately require a precise alignment and control of the interacting surfaces. Although gross surface alignment can be incorporated into the instrumental design, it is unfortunately much harder to control small-scale variations due to spatial differences in surface topography and chemistry. We believe that these latter factors are some of the remaining bottle-necks to the future progress of such force-based approaches.

To this end, researchers have sought methods to selectively immobilize biomolecules with enhanced control. For example, various surface chemistry approaches have been used to optimize the density of immobilized biomolecules, to remove steric hindrance between interacting biomolecules, and to avoid unwanted nonspecific binding and/or the formation of multiple biomolecular complexes.[22,23] While the covalent approach has shown clear advantages over the nonspecific adsorption approach, the commonly employed oxide−silane and gold−thiol chemistries[24,25] have yet to be fully optimized to meet the ultimate goal. Mixed self-assembled monolayers (SAMs), which are typically prepared through coadsorption of an active surfactant and an inactive one, have also been applied for biomolecular force measurements.[23] Mixed SAMs enable control of the density of surface functional groups, but unfortunately statistics still governs their distribution. Usually the practical situation is also often worse than predicted, because molecules with the similar functional groups tend to associate closely to form aggregates. Therefore, while the mixed SAM approach is reasonable, a new approach to overcome their known disadvantages would be welcomed.

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We have examined a more rational approach, controlling the spacing between the immobilized molecules through the self-assembly of dendrons on surface. Dendrons, conically shaped molecules of which repeating units are directionally stretched from a core, are highly branched polymers with uniform size and molecular weight as well as well-defined structure.[26] Because it is possible to control their size precisely and utilize their reactive termini for their effective self-assembly on the surface, they seem ideal building blocks for creating new materials of which the surface characteristics are finely tuned at the molecular level. In previous studies, our group successfully introduced a cone-shaped dendron to various oxide substrates.[27] The mesospacing provided by the cone shape naturally was found to yield an optimal DNA microarray, where each probe DNA strand leaves ample space for the incoming target DNA to interact, resulting in enhanced kinetics and a selectivity of single-nucleotide polymorphism detection which was as high as that observed in solution (100:1). Moreover, the observed high hybridization yield confirmed that DNA probes on the surface with ca. 3 nm spacing experienced minimal steric hindrance during the hybridization.

In this work, we examined the dendron immobilization approach for biomolecular force measurements. Through a series of force measurements recorded between interacting dendron surfaces functionalized with complementary oligonucleotides, we observed several unique properties of surfaces modified via this approach. We believe that the unique properties afforded to the surface by the dendron method predominantly relate to the provided mesospacing of the functional groups.

Materials and Methods

General. The silane coupling agent *N*-(3-(triethoxysilyl)propyl)-*O*-polyethyleneoxide urethane (TPU) was purchased from Gelest Inc. All other chemicals are of reagent grade from Sigma-Aldrich. The UV-grade fused-silica plates were purchased from CVI Laser Co. The polished prime Si(100) wafers (dopant, phosphorus; resistivity, 1.5−2.1 Ω·cm) were purchased from MEMC Electronic Materials Inc. Deionized water (18 MΩ·cm) was obtained by passing distilled water through a Barnstead E-pure 3-Module system.

Sample Preparation. 1. Cleaning the Substrates. Silicon wafers (and fused-silica plates for dendron surface coverage analysis; see the Supporting Information) were sonicated in Piranha solution (concentrated H_2SO_4/30% H_2O_2 = 7:3 (v/v)) for 4 h (**Caution:** *Piranha solution can oxidize organic materials explosively. Avoid contact with oxidizable materials.*). The substrates were washed and rinsed thoroughly with deionized water after sonication. Subsequently, they were immersed in a mixture of deionized water, concentrated ammonia solution, and 30% hydrogen peroxide (5:1:1 (v/v/v)) contained in a Teflon beaker. The beaker was placed in a water bath and heated at 80 °C for 10 min. The substrates were taken out of the solution and rinsed thoroughly with deionized water. Again, the substrates were placed in a Teflon beaker containing a mixture of deionized water, concentrated hydrochloric acid, and 30% hydrogen peroxide (6:1:1 (v/v/v)). The beaker was heated at 80 °C for 10 min. The substrates were taken out of the solution and washed and rinsed thoroughly with a copious amount of deionized water. The clean substrates were dried in a vacuum

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chamber (30−40 mTorr) for about 20 min and used immediately for the next steps.

2. AFM Probe Pretreatment. Standard V-shaped silicon nitride cantilevers (MLCT−AUNM) with pyramidal tips (Veeco Instruments; $k = 10$ pN/nm) were first oxidized by dipping in 10% nitric acid and heating at 80 °C for 20 min. The cantilevers were taken out of the solution and washed and rinsed thoroughly with a copious amount of deionized water. The clean cantilevers were dried in a vacuum chamber (30−40 mTorr) for about 20 min and used immediately for the next steps.

3. Silylation. Silicon/silica substrates and cantilevers pretreated as above to provide a thin silica top layer were immersed into anhydrous toluene (20 mL) containing the coupling agent (0.20 mL) under a nitrogen atmosphere and placed in the solution for 6 h. After silylation, the substrates and cantilevers were washed with toluene, then baked for 30 min at 110 °C. The substrates were immersed in toluene, toluene−methanol (1:1 (v/v)), and methanol in a sequential manner, and they were sonicated for 3 min in each washing solution. The cantilevers were rinsed thoroughly with toluene and methanol in a sequential manner. Finally, the substrates and cantilevers were dried under vacuum (30−40 mTorr). The experimental procedures for silylation with GPDES and subsequent opening of the epoxide with ethylene glycol are described elsewhere.[27]

4. Preparation of Dendron-Modified Surfaces. The above hydroxylated substrates and cantilevers were immersed into a methylene chloride solution with a small amount of DMF dissolving the dendron (1.0 mM) and a coupling agent, 1,3-dicyclohexylcarbodiimide (DCC) (9.9 mM) in the presence of 4-dimethylaminopyridine (DMAP) (0.90 mM) for 12−24 h. The dendron, 9-anthrylmethyl *N*-({[tris({2-[({tris-[(2-carboxyethoxy)methyl]methyl}amino)carbonyl]ethoxy}methyl)-methyl]amino}carbonyl)propylcarbamate used in this work was prepared in this group. After reaction, the substrates were immersed in methylene chloride, methanol, and water in a sequential manner, and they were sonicated for 3 min at each washing step. The cantilevers rinsed thoroughly with methylene chloride, methanol, and water in a sequential manner. Finally, the substrates and cantilevers were washed with methanol and dried under vacuum (30−40 mTorr).

5. Deprotection of 9-Anthrylmethoxycarbonyl Group. The dendron-modified substrates and cantilevers were immersed into a methylene chloride solution with 1.0 M trifluoroacetic acid (TFA), and they were stirred for 3 h. After the reaction, they were soaked in a methylene chloride solution with 20% (v/v) diisopropylethylamine (DIPEA) for 10 min. The substrates were sonicated in methylene chloride and methanol each for 3 min, and the cantilevers were rinsed thoroughly with methylene chloride and methanol in a sequential manner. The substrates and cantilevers were dried under vacuum (30−40 mTorr).

6. Preparing NHS-Modified Substrates. The above deprotected substrates and cantilevers were immersed into an acetonitrile solution with di(*N*-succinimidyl)carbonate (DSC) (25 mM) and DIPEA (1.0 mM) for 4 h under nitrogen atmosphere. After the reaction, the substrates and cantilevers were placed in stirred dimethylformamide for 30 min and washed with methanol. The substrates and cantilevers were dried under vacuum (30−40 mTorr).

7. Immobilization of Oligonucleotides. The above NHS-modified substrates and cantilevers were soaked in oligonucleotide solution (20 μM in 25 mM $NaHCO_3$ buffer (pH 8.5) with 5.0 mM $MgCl_2$) for 12 h. After the reaction, the substrates and cantilevers were stirred in a hybridization buffer solution (2× SSPE buffer (pH 7.4) containing 7.0 mM sodium dodecylsulfate) at 37 °C for 1 h and in boiling water for 5 min to remove nonspecifically bound oligonucleotide. Finally the substrates and cantilevers were dried under vacuum (30−40 mTorr).

AFM Force Measurements. All force measurements were performed with a NanoWizard AFM (JPK Instrument). The spring constant of each AFM tip was calibrated in solution before each experiment by the thermal fluctuation method. The spring constants of the cantilevers employed varied between 12 and 15 pN/nm. All measurements were carried out in a fresh PBS buffer (pH 7.4) at room temperature. Force curves were always recorded more than 100 times at one position on a substrate, and at least five spots examined in each separate experiment. It should also be noted that the experiment was repeated many times using different tips and samples, and the attractive/adhesion behavior reported was consistently reproduced.

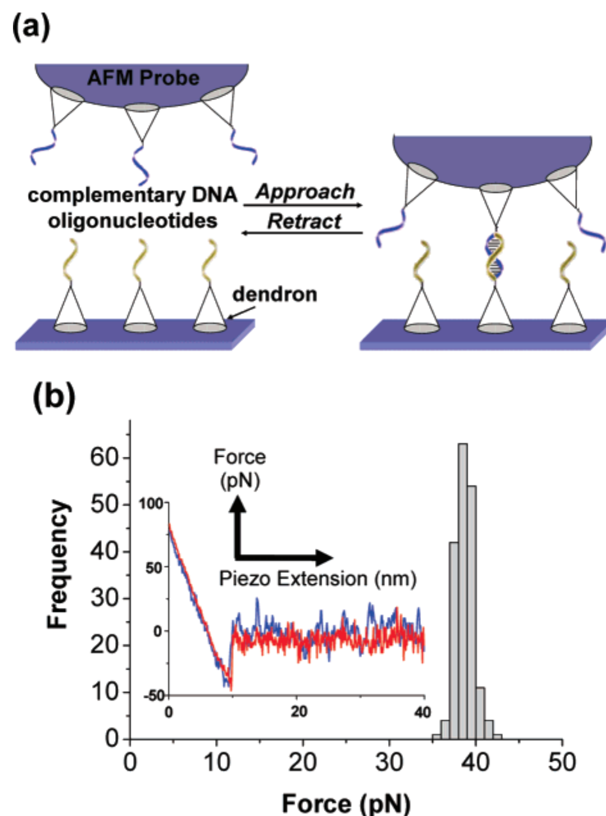

Figure 1. (a) Schematic of the experimental setup employed for the measurement described within this study. With oligonucleotide sequences (Table 1) immobilized on the dendron-modified surfaces, force measurements were recorded by bringing the probe and substrate into and out of contact. (b) A typical measurement (inset: approach and retract traces (red and blue curves, respectively)) and distribution of adhesive forces recorded for the complementary 30-mer sequence.

Results

Recording Forces between Functionalized Dendron Arrays. In all experiments, DNA oligonucleotides were covalently attached to silicon substrates or silicon nitride AFM tips via their 5′ termini using a modification of a dendron-based surface functionalization method that has been described previously.[27−29] Force versus distance measurements were then recorded as functionalized AFM tips and surfaces were brought into and out of contact (Figure 1). Attractive forces (binding forces) in their approach traces and adhesive forces (rupture forces) in their retraction traces were observed.

In previous studies, the dendron immobilization approach has been shown to increase the efficiency of conventional (non-force-based) DNA chip assays, most likely due to an increase in the uniformity of the surface attachment chemistry and a reduction in steric hindrance afforded by the mesospacing of the surface bound oligonucleotides.[27,30,31] It provides a close-

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Table 1. Summary of the Oligonucleotide Species Employed Throughout These Experiments[a]

	surface oligonucleotide	tip oligonucleotide (fully complementary)	tip oligonucleotide (single base mismatch)	tip oligonucleotide (double base mismatch)
20 bases	5′-H2N- CCATCGTGGTT GCTCCTCAG-3′	5′-H2N- CTGAGGAGCA ACCACGATGG-3′	5′-H2N- CTGAGGAGCT ACCACGATGG-3′	5′-H2N- CTGAGGAGCT TCCACGATGG-3′
30 bases	5′-H2N- GCTGCTATGG AGACACGCCC TGGAACGAAG-3′	5′-H2N- CTTCGTTCCAG GGCGTGTCTCC ATAGCAGC-3′	5′-H2N- CTTCGTTCCAG GGCGCGTCTCC ATAGCAGC-3′	5′-H2N- CTTCGTTCCAG GGCTCGTCTCC ATAGCAGC-3′
40 bases	5′-H2N- TGGATCTGGG GTGCCATTCCGCTGTCTCAAGG TGTGCTCG-3′	5′-H2N- CGAGCACACC TTGAGACAGC GGAATGGCAC CCCAGATCCA-3′	5′-H2N- CGAGCACACC TTGAGACAGC GTAATGGCAC CCCAGATCCA-3′	5′-H2N- CGAGCACACC TTGAGACAGC GTCATGGCAC CCCAGATCCA-3′
50 bases	5′-H2N- GTCTGACCTGT TCCAACGACC CGTATCACTCC GCTCCTGCCTG CTCTCCA-3′	5′-H2N- TGGAGAGCAG GCAGGAGCGG AGTGATACGG GTCGTTGGAA CAGGTCAGAC-3′	5′-H2N- TGGAGAGCAG GCAGGAGCGG AGTGTTACGG GTCGTTGGAA CAGGTCAGAC-3′	5′-H2N- TGGAGAGCAG GCAGGAGCGG AGTGTAACGG GTCGTTGGAA CAGGTCAGAC-3′

[a] The underlined parts indicate the mismatch sites, in the single and double base mismatch experiments.

packed array of protected amine functional groups with a spacing that can potentially be tailored depending on the particular dendron generation (i.e., level of branching) employed and to which the 5′ termini of oligonucleotides can be attached. The separation of such groups can be further modified according to the underlying surface chemistry or "base layer" employed for dendron−substrate attachment (Supporting Information).

Here, TPU was employed to generate the underlying silane base layer, to which the dendron (9-anthrylmethyl *N*-({[tris({2-[({tris[(2-carboxyethoxy)methyl]methyl}amino)carbonyl]ethoxy}methyl)methyl]amino}carbonyl)propylcarbamate) (or 9-acid) species was subsequently immobilized. The TPU base-layer chemistry was chosen rather than the glycidylpropyldiethoxymethylsilane (GPDES)-modified surfaces employed previously,[27] as it was found to consistently result in clean force curves with single pull-off events and narrow force distributions (Figure 1b and Supporting Information). Following immobilization of the dendron onto TPU substrates, and after the deprotection of the anthryl protecting group, the amine group was activated with DSC and an amine tethering oligonucleotide subsequently immobilized (Table 1 for oligonucleotide sequences).

Determining the Specificity of the Interaction Forces. First, to determine the specificity of the tip−sample interactions recorded for fully complementary sequences (Figure 1b), control measurements were recorded between tips and surfaces modified with noncomplementary oligonucleotides. In all cases, greater than 95% of the recorded force curves displayed no interaction, with the remaining measurements showing adhesion forces of less than 16 pN (Figure 2a). This is in marked contrast with measurements recorded between tips and surfaces functionalized with only the underlying silane and dendron surface chemistries, which displayed large adhesion forces (>1 nN) (data not shown). Similar large (>1 nN) adhesion forces were also recorded on dendron surfaces, in which only one of the interacting surfaces (e.g., tip or surface) was also functionalized with oligonucleotide (Figure 2b).

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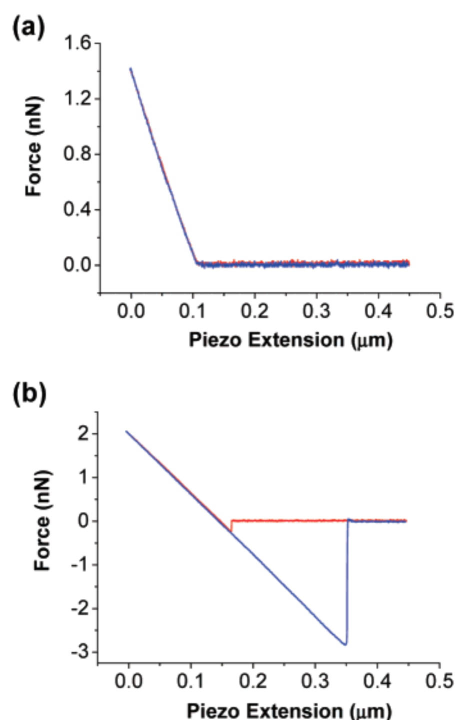

Figure 2. (a) Control measurements between dendron surfaces functionalized with noncomplementary oligonucleotides displayed no adhesion (or attraction) in >95% of measurements. (b) Measurements recorded between surfaces in which only the tip or substrate was functionalized with oligonucleotide resulted in large adhesive (and attractive forces). Measurements recorded between surfaces only functionalized with dendrons were of similar appearance. In both measurements the red and blue curves correspond to the approach and retract traces, respectively.

Dependency of the Forces on Base-Pair Number. To investigate how the forces recorded between complementary oligonucleotides were influenced by the number of bases a series of experiments were performed in which measurements were recorded between tips and surfaces functionalized with complementary sequences ranging from 20 to 50 base pairs (Table 1). In each experiment, greater than 80% of the measurements displayed adhesion traces with single clean pull-off events, and

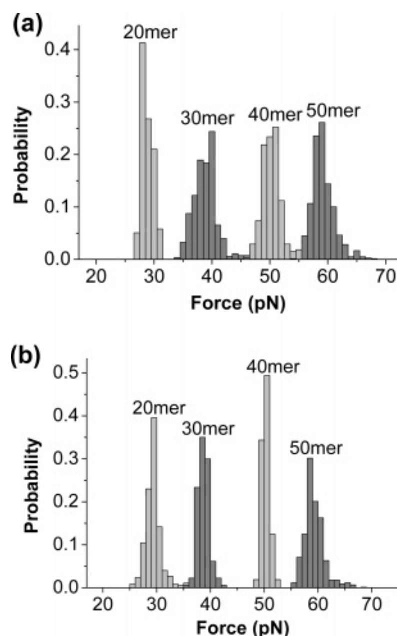

Figure 3. Distribution of (a) attractive and (b) adhesive forces recorded at 0.1 μm s^{-1} for all of the fully complementary sequences detailed in Table 1. The *y*-axis reflects the probability of observing a force of particular magnitude within each distribution.

produced force versus distance profiles that were similar in appearance to those presented in Figure 1b. Interestingly, at measurement rates >0.1 μm s^{-1} all measurements displaying adhesive events also presented with attractive peaks or "jumps-to-contact" in their approach traces, and only when the measurement rate was reduced to below 0.1 μm s^{-1} did the frequency of observing an attractive event decrease (falling to around 50% at 0.01 μm s^{-1}, although the frequency of observing adhesive unbinding events remained unchanged at this rate). It should also be noted that the control measurements recorded between noncomplementary oligonucleotide surfaces demonstrated significantly lower attractive forces in their approach traces, with a high proportion of these measurements showing no interaction.

Figure 3 shows the distributions of (Figure 3a) attractive and (Figure 3b) adhesion (unbinding) forces recorded at 0.1 μm s^{-1} for the complementary oligonucleotide sequences shown in Table 1. The first striking observation is the narrowness of the force distributions (the maximum half-width is ~3 pN) and the fact that both types of force are at approximately the same magnitude for a given sequence at the particular rate. The values of binding forces are 28, 40, 51, and 58 pN, and those of unbinding forces are 29, 38, 50, and 58 pN for 20, 30, 40, and 50 base pairs, respectively. Coincidently the increment of the force is roughly 10 pN at each increase of 10 DNA bases. These data therefore suggest that both the attractive and adhesive forces are dependent on the number of interacting bases, and hence that they originate from a phenomenon that is governed by specific oligonucleotide interactions.

Discussion

The magnitudes of the adhesion forces recorded for each complementary sequence are close to those reported for the rupture of single DNA and RNA duplexes formed from oligonucleotides of similar length.[11-13] Considering also the control experiments performed between surfaces at various stages in the immobilization process and with noncomplementary oligonucleotides, it is thus tempting to suggest that the observed forces originate from the formation/breakage of single oligonucleotide duplexes. However, several of our observations are not consistent with this scenario.

We first consider the ability to observe attractive forces or "binding events" in the approach traces of force measurements. While these observations are extremely interesting and novel, in particular as they appear to vary with the number of base pairs, the ability to measure single-molecule binding events in such measurements is fundamentally limited by the physical properties of a force transducer. For example, a soft AFM cantilever with stiffness around 10 pN nm^{-1} fluctuates with thermal energy (equivalent at room temperature to 4.11 pN nm) by several angstroms, and therefore produces a noise in force of around 10 pN. To detect an interaction above the noise, the binding event would therefore need to deflect the lever by several nanometers, a distance that is significantly longer than a single-molecule binding potential. It is therefore difficult to ascribe the observed attractive events to the binding of single oligonucleotides. Second, the high frequency of observation of attractive and adhesion events suggests that on average many interactions are formed and broken in each force measurement cycle.[32-34] While the sharpness of the obtained force distributions and force measurements is also impressive, unfortunately their shapes are inconsistent with those already documented for the measurement of single-molecule interactions.[32-34] Even for measurements recorded between small complementary oligonucleotides (such as those employed) one would expect to see a nonlinear profile in the retract trace prior to the rupture event, corresponding to the extension of the molecule and its underlying attachment chemistry. Previous single-molecule studies of the force-induced melting of DNA and RNA oligonucleotides[11-13] have in addition produced distributions with shapes that both agree with theory and are broader than those observed here. An alternative explanation is therefore required to describe the observed experimental observations.

We propose an alternative model in which, prior to surface contact, the tethered single olignucleotide strands act to form a negatively charged entropic steric barrier above the underlying substrates. The removal of the large attractive and adhesive forces between the underlying dendron surfaces upon subsequent functionalization with noncomplementary oligonucleotides is consistent with the introduction of such a barrier (Figure 2). When the opposing surfaces are functionalized with complementary oligonucleotides, the barrier will exist until a point where the surfaces are close enough that hybridization can occur. Upon hybridization, the repulsion provided by the barrier will be removed, causing the underlying surfaces to once again attract and collapse together.

In this model, the distance over which the surfaces move together will be comparable to the length of the barrier destroyed on hybridization, and hence the force that is measured should be proportional to the length of the duplexes formed. Figure 4 displays the dependence of the jump-in distance (calculated from the mode of the attractive "jump-in" force distributions and the

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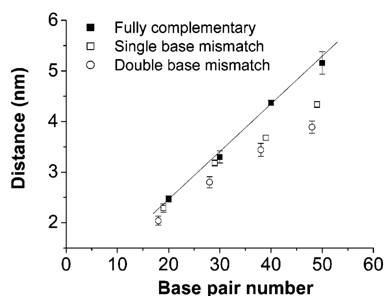

Figure 4. Dependency of the "jump-in" distance with the number of complementary base pairs. The distance is calculated from the attractive "jump-in" force divided by the average cantilever spring constant, 11.6 pN nm^{-1}. Filled squares indicate data obtained for the fully complementary sequences (each point represents the average mode attractive force observed in several experiments, with the bars representing the SD of these values), and the unfilled squares and circles represent the data obtained for the single and double base mismatch sequences, respectively.

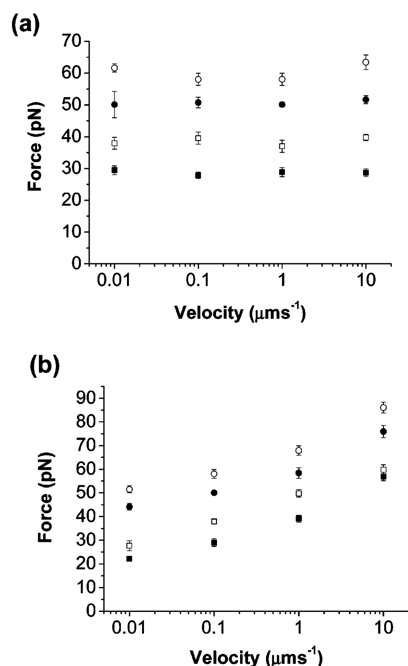

Figure 5. Dependency of the (a) attractive forces and (b) adhesive forces for the complementary DNAs on measurement speed (bars indicate the SD of the force data at each rate: filled square, 20-mer; open square, 30-mer; filled circle, 40-mer; open circle, 50-mer).

average cantilever spring constant of 11.6 pN nm^{-1}) with the number of complementary base pairs. The figure is composed from attractive force data recorded both with the same tip at different measurement velocities (Figure 5) and with different tips. The proportionality of the jump-in distance with base-pair number can clearly be observed, with the gradient revealing an average jump-in distance of 0.09 (SD 0.003) nm per base pair. An offset in the data of 0.6 (SD 0.1) nm is also revealed by the y-axis intercept, most likely attributed to a property of the underlying surface chemistry. Our measurements thus suggest that we are able to detect the length of hybridized oligonucleotides, primarily through the collapse of the repulsive barrier upon duplex formation.

To test this model, the rate dependence of the observed attractive and adhesive forces was investigated, as we predicted that the "barrier collapse" would be relatively insensitive to measurement speed, in marked contrast with measurements corresponding to the formation and rupture of single molecules (or several multiples thereof).[11,13,34,35] Figure 5 summarizes the data obtained at approach and retract speeds in the range of 0.01−10 μm s^{-1} for each complementary oligonucleotide sequence.

Dynamic force spectra of oligonucleotide dissociation presented by Strunz et al.[11] exhibited slopes of force versus loading rate (the *force scale*) that were inversely proportional to the strand length. As predicted by theory, the displacement of the energy barrier to strand dissociation was found to be linearly correlated and the extrapolated force-free dissociation rate found to be exponentially related to the number of base pairs broken. The spectra presented in Figure 5b show none of these behaviors; the rupture forces appear to increase with speed in a fashion that is independent of strand length. The increase in force also appears to be linearly dependent on the retract velocity and not logarithmically as known for single-molecule dissociation. The shapes of the rupture force distributions (Figure 3b) are also inconsistent with those single-molecule measurements of strand dissociation.

The apparent leveling off of the rupture forces at low retract speeds is, however, reminiscent of the force spectra predicted for force rupture studies of single-molecules systems close to force equilibrium.[36] The lowest modal force measured for each oligonucleotide is also similar to, although interestingly consistently lower than, the force measured on approach. So were we indeed measuring association and dissociation of single oligonucleotides? At low force loading rates the most probable rupture force is predicted to be a constant $f^{\otimes}$ that depends on the equilibrium binding/unbinding rates ($K_{eq} = k_{on}/k_{off}$) and the combined stiffness of the cantilever and molecular system, κ_s, by

$$f^{\otimes} \sim [2k_BT\kappa_S \ln(K_{eq})]^{1/2}$$

The doubling of the plateau force measured from studying 20−50 base pairs would, however, correspond to an unrealistic increase of the equilibrium constant. The forces measured from separation of the DNA immobilized surfaces are thus not consistent with single-molecule events. They are also at odds with those measured for multiple interactions loaded concurrently.

If measured close to equilibrium we should be able to apply the Crooks fluctuation theorem to determine the equilibrium free energy difference between duplex and single-strand states.[37] The limited force sensitivity and temporal resolution of the AFM precludes an accurate analysis, however, but we can approximate the work done on the lever of stiffness K in measuring a force F as $F^2/2K$. Taking the value of force where the binding and unbinding distributions overlap as the average of the modal force for binding and unbinding at 0.1 μm s^{-1} we obtain approximate values for equilibrium free energy difference as 7, 14, 23, and 30 k_BT for the 20-, 30-, 40-, and 50-mer, respectively. The resulting correlation between duplex length n and free energy of hybridization W of 0.8 k_BT-per-base-pair (i.e., $W \approx 0.8n$ −

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9) appears reasonable; however, the “missing” 9 k_BT and the insensitivity of the measured binding forces with approach speed are difficult to reconcile.

The data do, however, highlight the dependency of both the attractive and adhesive forces on the number of complementary base pairs. Importantly, they also demonstrate the insensitivity of the attractive forces to measurement speed, thus providing support for our proposed model. Interestingly, it can also be seen that while the attractive forces are relatively insensitive to this parameter, the adhesive forces increase with the speed of the measurement. As already discussed, the adhesive forces clearly vary in a nonlinear manner with the logarithm of the measurement speed, a behavior that is inconsistent with that previously observed for the forced unbinding of single DNA/RNA oligonucleotide duplexes. While it is difficult to provide a precise explanation for the observed trend, it is likely to arise through a reverse of the above combined with the rupture of multiple DNA duplexes. The fact that the magnitudes of the adhesive forces are also close to those already reported for single molecules suggests a complex unbinding mechanism, with the possible involvement of partial oligonucleotide overlaps in addition to the fully aligned sequences.

The data clearly demonstrate that both the attractive and adhesive forces are strongly dependent on the number of complementary bases. The finding that we are able to observe base-dependent attractive or “binding” forces is also intrinsically novel and opens up the exciting possibility of exploiting this phenomenon for screening applications. The attractive forces offer considerable potential for such applications as they are observed in greater than 80% of measurements and their magnitudes are distributed around a narrow range. It is intriguing to find that the force distributions become narrow when the spacing between DNAs is controlled properly. The narrowness of the force distributions would be particularly important if, for example, the screening of nucleic acid hybridization events and the discrimination of base mismatches were required. The low sensitivity of the attractive forces to measurement speed also offers significant practical advantages for future screening technologies, removing the need to optimize this parameter between instruments and user laboratories.

To investigate further the sensitivity of this approach, forces were recorded also for sequences with single base and double base mismatches (Table 1). For both types of sequence the introduction of the mismatch was found to decrease both the attractive and adhesive forces. For the single base mismatched pairs, attractive forces of 26, 37, 42, and 50 pN were recorded for the 20-, 30-, 40-, and 50-mer sequences, respectively, and for the double base mismatched sequences, forces of 24, 32, 40, and 46 pN were observed (Figure S2 in the Supporting Information for the single and double base mismatched sequence force distributions).

This mismatch data is included in Figure 4 for comparison to the fully complementary data. For the single base mismatch data, while the 20- and 30-mer data fit well to the linear relationship described by the fully complementary data, i.e., they fall at the distances predicted for 19 and 29 base pairs, it is clear that the data for the longer single base and double base mismatch sequences do not. These findings in fact suggest that the introduction of the mismatch alters the properties of the barrier and/or the formed duplexes so that the jump-in distance is no longer proportional to the number of complementary bases. However, while more detailed investigations are clearly needed to explore the basis of this change in behavior, the current data nevertheless indicate that we are able to discriminate between sequences with single base-pair resolution using this approach.

Conclusions

We have shown how nanoscale-engineered dendron surfaces comprising arrays of complementary DNA oligonucleotides can provide measurable forces of attraction and adhesion that relate to hybridization events. Importantly, we have been able to use this system to detect attractive and adhesive forces that can discriminate between duplexes of length differing by 10 base pairs and have shown that our measurement is also sensitive to single and double base-pair mismatches.

The ability to detect attractive forces that relate to biomolecular recognition events, rather than the more conventionally used adhesion forces is particularly novel. The findings that they can be observed in a high proportion of measurements, that they are distributed around a narrow range of values, and their lack of sensitivity to measurement rate also present considerable opportunity for future screening applications. For the surfaces examined thus far, our data suggests that these properties arise from the collapse of a steric barrier upon hybridization, which exists due to the close surface packing of the immobilized DNA molecules. We propose that as such properties should scale in a highly predictable manner (i.e., with dendron generation) they should also be readily adaptable to other molecular systems, e.g., for the study of DNA−RNA, DNA−protein, and RNA−protein interactions. For force-based detection methodologies, the described dendron method thus not only provides a route to obtaining controlled surface functionalization but also offers a new mode for the detection of specific molecular recognition events, a method that reflects the ensemble properties of the surface, and that only requires force as a reporter of binding. Additional reporters, such as fluorescence, are not required.

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Supporting Information Available: Influence of base-layer chemistry and dendron generation and single and double base mismatch data in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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