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Monitoring DNA Immobilization and Hybridization on Surfaces by Atomic Force Microscopy Force Measurements

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DNA immobilization and hybridization was carried out on Au substrates that were modified with mercaptopropanoic acid and then treated with aluminum(III) solution. The positively charged Al(III) film can be used to immobilize both ds-DNA and ss-DNA. Atomic force microscopy (AFM) was used to monitor the process by force measurements between a negatively charged silica tip and the substrates while immersed in dilute electrolyte. Surface hybridization of ss-DNA produces an increase in the surface charge and surface potential of the substrates, which is reflected by the increasing repulsive force as determined from AFM force−separation curves. A single-base mismatch was detectable in surface hybridization. The AFM force measuring technique was also employed to investigate the interaction of $Ru(phen)_3^{2+}$ with ss-DNA and ds-DNA. The force measurement results showed that there is a small interaction between $Ru(phen)_3^{2+}$ and ss-DNA, which was ascribed to the electrostatic binding of $Ru(phen)_3^{2+}$ to the ss-DNA surface. For ds-DNA, there is a strong interaction which is believed to be due to the association or intercalation of $Ru(phen)_3^{2+}$ with ds-DNA.

Recently, DNA diagnostics has become an important area in biotechnology and clinical medicine. The immobilization and hybridization of DNA on surfaces is of great interest in many applications.[1−4] Currently, DNA detection techniques include radiochemical,[5] enzymatic,[5] fluorescent,[5] and electrochemiluminescent methods,[6] field effect detection,[7] surface plasmon resonance spectroscopy,[8] electrochemical methods,[9,10] quartz crystal microbalance,[11] and atomic force microscopy (AFM) imaging methods.[12] Recently, two kinds of force measurements, AFM force measurements[13,14] and direct surface stress measurements,[15] have also been used to study DNA hybridization. A major advantage for force measurements to study DNA immobilization and hybridization is that it does not require labeling the molecules under investigation.

In an alternative AFM detection method, the AFM cantilever is modified with a silica sphere,[16,17] and the deflection of the cantilever is measured as a function of its separation from a surface. Compared to direct surface stress measurements of cantilevers, there are some advantages in force−separation curve measurements. One is that quantitative surface potential/surface charge information can be obtained by fitting an experimental force−separation curve to a theoretical curve employing the nonlinear Poisson−Boltzmann equation. Another is that while drift is frequently a serious problem in direct surface stress experiments, it can be greatly reduced in the force−separation curve experiments.

In a previous study from this laboratory,[18] we demonstrated that an aluminum(III) alkanebisphosphonate film can be used to immobilize both single-strand (ss) and double-strand (ds) DNA based on the interaction of the film metal center (Al^{3+}) with the phosphate group of DNA and the immobilized DNA could be determined by AFM force measurements. In this report, we show that an aluminum(III) carboxylate film can also be used to immobilize DNA. After immobilization, the film can be employed to recognize a complementary strand of DNA in solution. Force measurements clearly show the successful immobilization and hybridization of DNA on such surfaces. Moreover, the AFM force measuring technique was also employed to investigate the interaction of $Ru(phen)_3^{2+}$ with ss-DNA and ds-DNA.

EXPERIMENTAL SECTION

Materials. Mercaptopropanoic acid (Aldrich), $Al(NO_3)_3$ (Spectrum Chemical MFG), $Ru(phen)_3Cl_2 \cdot 6H_2O$(Aldrich), and $KClO_4$ (Aldrich) were used as received without further purification.

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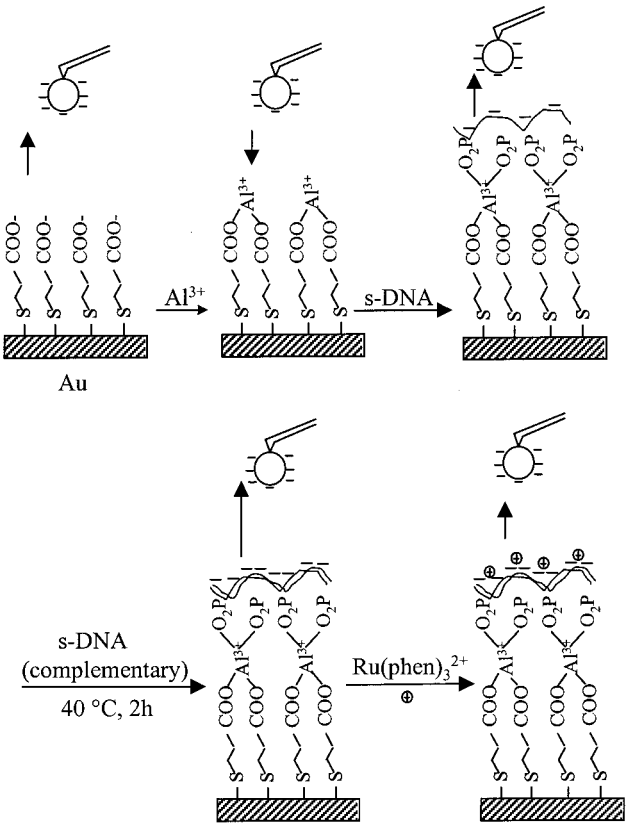

Figure 1. Schematic representation of the formation of aluminum carboxylate film, DNA adsorption and hybridization on gold substrates, and the interaction of $Ru(phen)_3^{2+}$ with the immobilized DNA and force curve measurements.

Solutions were prepared in 18-MΩ deionized water (Milli-Q Plus, Millipore Corp., Bedford, MA). The calf thymus ds-DNA and ss-DNA were purchased from Sigma. All other 10-base DNA samples, $poly(dA)_{10}$, $poly(dT)_{10}$, 5′-AATTCGGCAG-3′, 5′-TTAAGCCGTC-3′ and 5′-TTAAGCCGTG-3′ were purchased from Operon Technology Inc. (Alameda, CA).

Surface Modification. A schematic diagram for the surface modification is given in Figure 1. Gold substrates were prepared by vacuum evaporation of high-purity gold (99.999%) onto a cleaned silicon(100) wafer that was precoated with chromium to improve adhesion (typically, 200 nm of Au, 10 nm of Cr). The gold substrates were cleaned with piranha solution (98% H_2SO_4/30% H_2O_2, 4:1 v/v) prior to use. The self-assembled monolayer (SAM) of mercaptopropanoic acid was formed by immersing the gold substrates into a 5 mM mercaptopropanoic acid solution in ethanol for 12 h. After successive rinsing with ethanol and water, the SAM-coated substrate was then immersed in 5 mM $Al(NO_3)_3$ for 2 h.

DNA Immobilization and Hybridization. Calf thymus ds-DNA and ss-DNA were dissolved in 5 mM tris buffer and 50 mM NaCl overnight. The base concentrations of ds-DNA and ss-DNA determined by UV absorbance were 1 mM and 56 μM, respectively. In other cases, 10-base ss-DNA was dissolved in 5 mM tris buffer and 50 mM NaCl with the base concentration of 50 μM.

DNA immobilization was obtained by immersing the Al-terminated substrates in DNA solution for 2 h. The temperature and time for hybridization was 40 °C and 2 h in a buffer solution of 5 mM tris and 50 mM NaCl.

Interaction of Immobilized DNA with $Ru(phen)_3^{2+}$. The DNA immobilized substrate was soaked in a 1 mM $Ru(phen)_3Cl_2$ aqueous solution for 30 min, rinsed with H_2O, dried with argon, and then put in the AFM liquid cell for force measurements.

AFM Force Measurement and Data Analysis. All force measurements were performed with a Nanoscope III AFM (Digital Instruments) equipped with a piezoscanner having a maximum scan range of 15 μm ×15 μm × 2 μm. The standard AFM silicon nitride tip was modified by the attachment of a spherical silica bead. The AFM force measuring technique is well documented,[17,19,20] and the experimental details have been described elsewhere.[19] The diameters of the silica spheres used were 10−16 μm. The spring constant of the silica sphere modified cantilever was 0.35−0.65 N/m. During the acquisition of a force curve, cantilever deflections were monitored by recording the changes in voltage in a split photodiode, onto which was focused a laser beam reflected from the backside of the cantilever. The *z* direction displacement is given by the piezoscanner voltages. The raw data were converted to a normalized force/radius (F/R) versus tip−substrate separation for further analysis with knowledge of the scanner calibration, cantilever spring constant, and tip radius. The force was obtained by multiplying the deflection of the cantilever with its spring constant, and the tip−sample separation was calculated by adding the deflection to the position.[19,21]

Derjaguin−Landau−Verwey−Overbeek (DLVO) theory[22−25] was employed to calculate the surface potential. The electrical double-layer interaction energy was calculated for the constant-charge limit of the complete nonlinear Poisson−Boltzmann equation using the method of Hillier et al.,[19] who used a finite element discretization of the equation with linear basis functions. The Hamaker constants used for the theoretical calculations were 0.88×10^{-20} [26,27] and 1.2×10^{-19} J[19] for the silica−silica and silica−gold interactions, respectively. The surface potential of the silica sphere, determined, as in previous studies,[19] by force measurements above a silica substrate, was −40 mV.

RESULTS AND DISCUSSION

Aluminum Carboxylate Thin Film and DNA Immobilization. The immobilization of DNA on surfaces is of key importance in studies of DNA and in various applications. Basically, an ideal immobilization strategy should satisfy the following requirements: (1) a single strand of DNA must be strongly attached onto the surface; (2) hybridization of an immobilized DNA strand with a complementary DNA strand in solution is possible and adequate; (3) nonspecific adsorption of a noncomplementary DNA strand on the surface is negligible; (4) the modified layer for immobilizing DNA should be stable under further experiments and characterization; and (5) the immobilization strategy should be simple and easy to handle.

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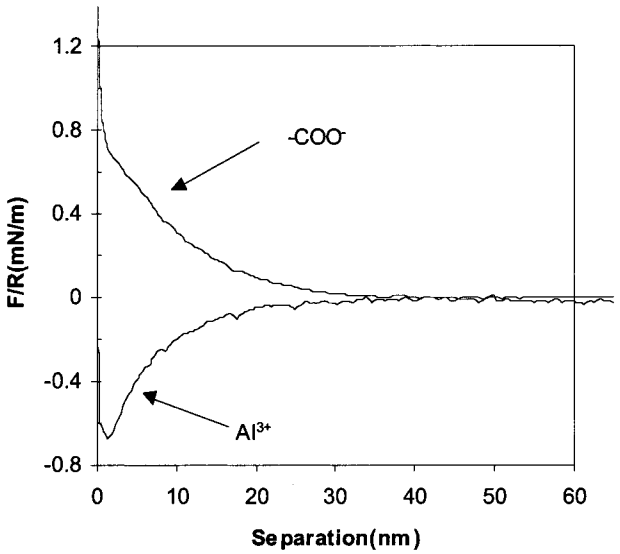

Figure 2. Forces obtained in a 1 mM $KClO_4$ aqueous solution of pH 5.5 between a silica probe and the thiol-derivatized carboxylate-terminated gold substrate before (upper curve) and after (lower curve) immersing the substrate into the aluminum(III) aqueous solution.

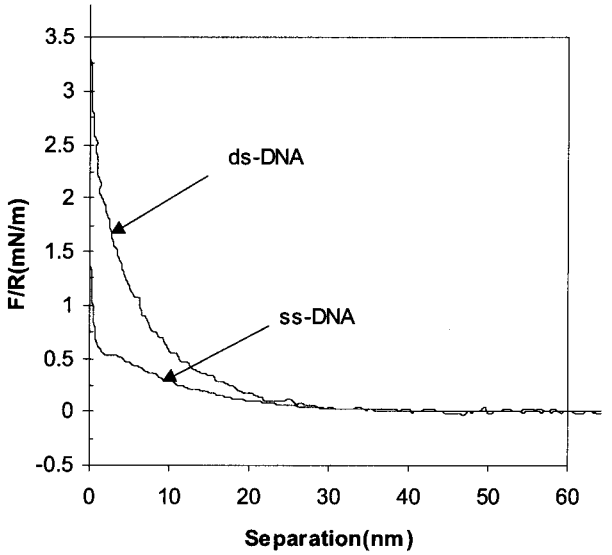

Figure 3. Forces obtained in a 1 mM $KClO_4$ aqueous solution of pH 5.5 between silica probes and thiol-derivatized carboxylate-terminated gold substrates after immersing the substrates into calf thymus ds-DNA and ss-DNA solutions.

In previous work,[18] we showed that the electrostatic force between a positively charged Al(III) surface and the negatively charged phosphate backbone of a DNA strand could be used to immobilize DNA. In the present work, we use a similar strategy to bind Al^{3+} with mercaptopropanoic acid, which is commercially available and has a comparable Al^{3+} binding ability as phosphonate.[28]

The force−separation curve for a silica probe and the mercaptopropanoic acid-modified Au substrate is depicted in Figure 2. There are a number of literature citations for the acid−base properties studies of acid-terminated SAMs.[29−33] The reported surface pK_a value for a mercaptocarboxylate film ranges from 5.2 to 7.7. In this study, we also carried out force titration measurements to measure the surface pK_a and obtained a value of 5.4. Under the given experimental conditions (10^{-3} M $KClO_4$ solution at pH 5.5), a repulsive force is obtained. Taking into account that the silica tip is negatively charged at this pH, the repulsive force means the surface is negatively charged. That is, under these conditions the acid-terminated monolayer is deprotonated to some extent.

To calculate the surface electrostatic potentials of the surface of interest, the force data were compared to theoretical predictions determined by solving the complete nonlinear Poisson−Boltzmann equation for forces between dissimilarly charged surfaces.[19] Traditionally, surface charge can also be obtained from surface potential using the following equation:[34]

$$\sigma^{M} = -\sigma^{S} = (8kT\epsilon\epsilon_0 n^0)\sinh(ze\psi_0/2kT)$$

Here, ϵ is the dielectric constant of the electrolyte solution, n^0 is the bulk concentration, ψ_0 is the surface potential, and z is the charge number of the electrolyte. The other symbols have their usual meaning. However, in recent work[35,36] using in situ AFM and electrochemical measurements of charge, a significant difference between the surface charge obtained from electrochemical methods and those obtained using AFM was found. The surface charge from AFM, the "effective surface charge", was much less than the real surface charge from electrochemistry. Because of this ambiguity in AFM charge measurements at electrodes, we mainly use the measured surface potential in discussing the experimental results in what follows.

After the gold substrate was immersed in 5 mM $Al(NO_3)_3$ for 2 h, force measurements in the same solution (10^{-3} M $KClO_4$ solution, pH 5.5) produced an attractive force (Figure 2), showing that the gold substrate became positively charged. The corresponding surface potential changed from -62 ± 2 to 10 ± 1 mV. As noted earlier,[18] aluminum(III) ions bound to the surface render the surface positive and result in the observed charge reversal on the surface of the substrate.

Aluminum carboxylate films, prepared as described above, were immersed in different solutions of 1 mM (base pair concentration) calf thymus ds-DNA and 56 μM (base pair concentration) calf thymus ss-DNA. Figure 3 shows the results of the force−separation curves for ds-DNA and ss-DNA. An electrostatic repulsive force was obtained, indicating a change in the surface from a positively charged state to a negatively charged one. This change, as shown previously, can be attributed to the immobilization of DNA, which left some excess phosphate groups.

(28) We also tried another commercialy available molecule (mercaptopropanoic sulfanoic acid) and some other metal ions (Zn^{2+} and Ca^{2+}); we found that only surface-bound short-chain carboxylate binds Al^{3+} well enough to obtain the needed positively charged surface for DNA immobilization.

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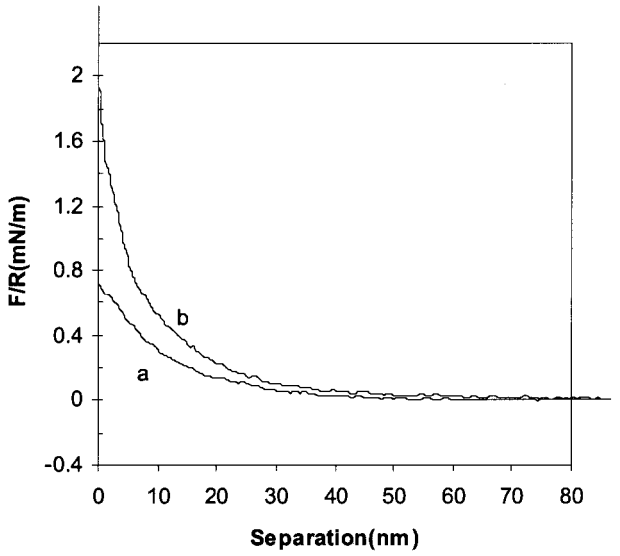

Figure 4. Forces between a silica probe and poly(dA)$_{10}$-immobilized gold substrate in 1 mM $KClO_4$ aqueous solution (a) before and (b) after hybridizing with poly(dT)$_{10}$.

Figure 3 shows that both ds-DNA and ss-DNA can be immobilized on an aluminum carboxylate film. However, the force for ds-DNA is much larger than for ss-DNA (the corresponding surface potentials are -62 ± 5 and -38 ± 2 mV), which suggests it may be possible to distinguish ss-DNA and its hybridized ds-DNA by force−separation curves. This is the key issue of this work.

The immobilization of DNA is a prerequisite for DNA hybridization on surfaces. To immobilize DNA on surfaces, one often needs to modify the DNA molecule, for example, by thiol derivatization.[37−41] Recently, DNA was shown to adsorb onto cationic lipid bilayers on mica[42] and gold[43] substrates. In our previous work[18] and in this work, DNA was immobilized and then hybridized to its complementary strand on a positively charged Al(III) surface. The advantage of using this method to immobilize DNA is that one does not need to modify the DNA molecule. Of course a problem with this approach is the necessity to deal with some adsorption of ss-DNA.

DNA Hybridization. The first demonstration of the hybridization of ss-DNA was performed with a strand of DNA containing a single base (A). The aluminum carboxylate film, prepared as described above, was immersed in 50 μM [NP] poly(dA) solution for 2 h. Figure 4a shows the force−separation curve for poly(dA), which is very similar to the curve for calf thymus ss-DNA. A repulsive force curve was obtained and the surface potential was −40 mV. The negatively charged surface confirms the successful immobilization of poly(dA) onto the aluminum carboxylate film surface.

Surface hybridization is a key step for most analytical applications of DNA. There are many factors that affect surface hybridization, e.g., surface coverage, buffer concentration, hybridization temperature, and hybridization time.[44] To obtain good surface hybridization, it is important to optimize the experimental conditions. In this work, our optimized hybridization conditions were as follows: 5 mM tris buffer solution with 50 mM NaCl, heated at 40 °C for 2 h. Figure 4b shows the force−separation curve for immobilized poly(dA) after its hybridization with poly(dT) under the above experimental conditions. As one can see, there is a significant increase in the force after hybridization. Overall, the surface potential increased from −40 V to −60 mV, indicating a greater negative charge upon hybridization.

It is interesting to know the origin of the increasing surface charge upon hybridization. As noted above, in ss-DNA, the repulsive force comes from the interaction of the negatively charged silica tip and the negatively charged phosphate groups of the DNA. After the immobilized ss-DNA is hybridized with the complementary strand (cs-) DNA in the solution, the density of DNA on the surface increases so the density of negatively charged phosphate groups also increases. This led to the increased (negative) surface charge, and therefore, the increased force between the silica tip and substrate. The importance of the above deduction is straightforward: because the surface charge will increase upon hybridization, it is possible to monitor surface immobilization and hybridization by surface charge/surface potential detection. AFM force measurement is a direct measurement of the interaction of two surfaces. Because surface potential/surface charge information can be obtained by fitting the force curve by solving the nonlinear Poisson−Boltzmann equation, we can monitor surface immobilization and hybridization of DNA using AFM force measurements.

To further test the validity of surface hybridization detected by AFM force measurements, another experiment was performed. A 10-base ss-DNA, 5′-AATTCGGCAG-3′, was used and hybridized with cs-DNA, 5′-TTAAGCCGTC-3′. The immobilization and hybridization conditions were the same as that for poly(dA). Figure 5 shows the force−separation curves for 5′-AATTCGGCAG-3′ before and after hybridization with 5′-TTAAGCCGTC-3′. A result similar to poly(dA) was obtained. For single-strand 5′-AATTCGGCAG-3′ DNA (Figure 5a), there was a repulsive force curve with a surface potential of −60 mV. After hybridizing with 5′-TTAAGCCGTC-3′ (Figure 5b), the repulsive force greatly increased, with a surface potential of −120 mV. Clearly, this result proves again the validity of using AFM force measurements to monitor surface hybridization of DNA.

Nonspecific adsorption can be a serious problem in DNA studies. Noncomplementary DNA strands also tend to adsorb onto surfaces previously exposed to ss-DNA. The effect of nonspecific adsorption and hybridization is similar, which makes nonspecific adsorption a serious interference for DNA hybridization detection. A good analytical method for DNA hybridization detection should avoid or reduce nonspecific adsorption as much as possible. A crucial test is whether a single-base mismatch between two DNA sequences can be detected. This was investigated by immersing the 5′-AATTCGGCAG-3′ immobilized substrate into the solution of 5′-TTAAGCCGTG-3′, which is a one-base mismatch with 5′-TTAAGCCGTC-3′, the cs-DNA of 5′-AATTCGGCAG-3′. Figure 5c shows the force−separation curve. The force for 5′-AATTCG-

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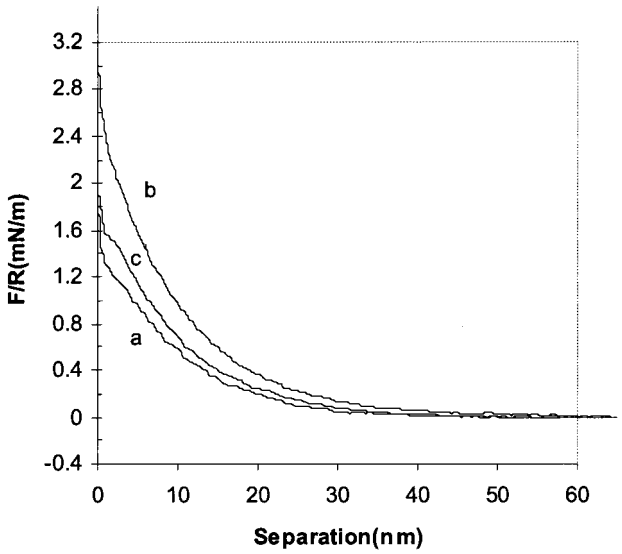

Figure 5. Forces between a silica probe and 5′-AATTCGGCAG-3′-immobilized gold substrate in 1 mM $KClO_4$ aqueous solution (a) before and (b) after hybridizing with 5′-TTAAGCCGTC-3′. Curve c shows the interaction of 5′-AATTCGGCAG-3′ with the one-base mismatch cs-DNA 5′-TTAAGCCGTG-3′.

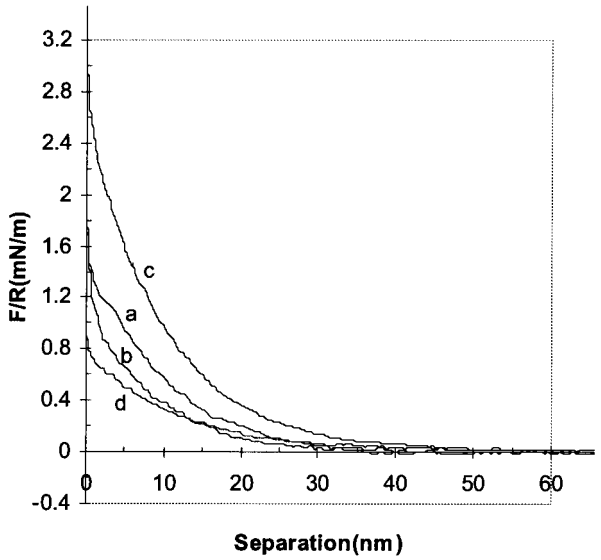

Figure 6. Forces between a silica probe and DNA-immobilized gold substrate in 1 mM $KClO_4$ aqueous solution: ss-DNA(5′-AATTCG-GCAG-3′) (a) before and (b) after interacting with $Ru(phen)_3^{2+}$; ds-DNA(5′-AATTCGGCAG-3′/5′-TTAAGCCGTC-3′) (c) before and (d) after interacting with $Ru(phen)_3^{2+}$.

GCAG-3′/5′-TTAAGCCGTG-3′ is larger than single-strand 5′-AATTCGGCAG-3′ but smaller than 5′-AATTCGGCAG-3′/5′-TTAAGCCGTC-3′. This potential, −80 mV, falls between the surface potential for 5′-AATTCGGCAG-3′ (−60 mV) and that for 5′-AATTCGGCAG-3′/5′-TTAAGCCGTC-3′ (−120 mV). This result shows that although there may be some nonspecific adsorption in the one-base mismatch case, one may still distinguish between hybridization and nonspecific adsorption.

Reproducibility is a key issue for analytical applications. We tested the reproducibility of our systems with five different samples for each case. For the ss-DNA, 5′-AATTCGGCAG-3′, the surface potential is 60 ± 5 mV. After hybridizing with 5′-TTAAGCCGTC-3′, the surface potential was 119 ± 6 mV. For the single-base mismatch case (5′-TTAAGCCGTG-3′ interacting with 5′-AATTCGGCAG-3′), the surface potential was 69 ± 9 mV. These results show that the reproducibility is quite good for these systems.

Interaction of Immobilized DNA with $Ru(phen)_3^{2+}$. The interaction of transition meal chelates with DNA is of great interest and has been the subject of a number of investigations.[45−50] Most studies focused on the interaction between the chelate and DNA in solution, with fewer concerning the association process between the chelate and surface-immobilized DNA. In a previous study,[18] preliminary results showed that AFM force measurements could be used to study the interaction between $Ru(phen)_3^{2+}$ and surface-immobilized DNA. Figure 6 shows the force−separation curve for 5′-AATTCGGCAG-3′ and 5′-AATTCGGCAG-3′/5′-TTAAGC-CGTC-3′ before and after interacting with $Ru(phen)_3^{2+}$. For 5′-AATTCGGCAG-3′, there is a small decrease of force after interacting with $Ru(phen)_3^{2+}$. The surface potential changed from −60 ± 5 to −43 ± 1 mV. For 5′-AATTCGGCAG-3′/5′-TTAAGC-CGTC-3′, there is a large decrease in force after interacting with $Ru(phen)_3^{2+}$. The surface potential changes from 119 ± 6 to −40 ± 3 mV. The result for the ds-DNA 5′-AATTCGGCAG-3′/5′-TTAAGCCGTC-3′ agrees with our previous results; that is, $Ru(phen)_3^{2+}$ can associate with ds-DNA, which leads to a large fraction of the negatively charged phosphate of the DNA being compensated. Therefore the force decreased greatly. For the ss-DNA 5′-AATTCGGCAG-3′, however, there is a slight difference when compared with previous results.[18] In this work, the surface potential for ss-5′-AATTCGGCAG-3′ also showed a slight decrease after interacting with $Ru(phen)_3^{2+}$. That means there is also a weak association between $Ru(phen)_3^{2+}$ with ss-5′-AATTCGGCAG-3′. There are different modes for the interaction between molecules and DNA, e.g., intercalation, electrostatic binding, binding in the minor or the major groove, and triple-helix formation.[51] Since surface-immobilized ss-DNA is negatively charged and $Ru(phen)_3^{2+}$ is positively charged, there is probably some electrostatic binding between the positively charged $Ru(phen)_3^{2+}$ and the negatively charged DNA surface. This electrostatic effect partially compensates the negative charge of phosphate ions of DNA and leads to a slight decrease in the force between the silica tip and DNA substrate.

CONCLUSION

A simple method was used for DNA immobilization and hybridization on surfaces. Au substrates were first modified with mercaptopropanoic acid and then treated with aluminum(III) solution. The positively charged Al(III) film can be used to immobilize both ds-DNA and ss-DNA. AFM force measurements

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showed there was a great increase in the repulsive force for the DNA substrates after hybridizing with cs-DNA in solution. This increased force is due to the increase of surface charge because of the increased density of negatively charged phosphate after surface hybridization. A single-base mismatch is also detectable in surface hybridization, which means the nonspecific adsorption does not present serious interference. The AFM force measuring technique was also employed to investigate the interaction of Ru(phen)$_3^{2+}$ with ss-DNA and ds-DNA. The force measurement results showed that there is a slight interaction between Ru(phen)$_3^{2+}$ and the ss-DNA, which was ascribed to the electrostatic binding of Ru(phen)$_3^{2+}$ onto the ss-DNA surface. On the other hand, for ds-DNA, there is a strong interaction, which is believed to be due to the intercalation of Ru(phen)$_3^{2+}$ with ds-DNA.

The above results suggest AFM force measurement may be a useful technique to study DNA immobilization and hybridization on surfaces. If spatial DNA array samples can be prepared, it may be possible to make use of the high spatial resolution of AFM and detect complementary and noncomplementary DNA strands simultaneously by force imaging. The complexity of current AFM instruments and the difficulty in scanning substrates immersed in liquids over distances larger than 150 μm, however, makes the application of AFM imaging problematical for actual multielectrode array samples. However, an alternative approach to cantilever-based AFM may make such scanning possible.

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