

Monitoring Molecular Beacon/DNA Interactions Using Atomic Force Microscopy

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The molecular beacon (MB) is a new fluorescence probe containing a single-stranded oligonucleotide with a probe sequence embedded in complementary sequences that form a hairpin stem. Due to the inherent fluorescent signal transduction mechanism, an MB functions as a sensitive probe with a high signal-to-background ratio for real-time monitoring and provides a variety of exciting opportunities in DNA, RNA, and protein studies. To better understand the properties of MBs, the specific interactions between MB and target DNA (complementary and one-base mismatch) have been directly investigated by atomic force microscopy. The interaction force between a linear DNA probe and the target DNA was also detected and compared to that between MB and target DNA. The results demonstrate the high specificity of the MB/target DNA compared to the linear DNA/target DNA interaction.

In the postgenomic era, quantitative studies of genomic information for drug discovery, as well as disease diagnosis and prevention, will be fast-growing areas of research and development. Growth in these areas has already produced a strong demand for advanced biomolecular recognition probes with high sensitivity and excellent specificity. One such promising probe for quantitative genomic studies is the molecular beacon (MB), a single-stranded DNA (ssDNA) molecule composed of a hairpin-shaped oligonucleotide that contains a fluorophore and a quencher group.^{1,2} A molecular beacon acts like a switch that is normally closed. When the fluorophore and quencher are spatially close, fluorescence is not observed (OFF state). But when the probe encounters a target DNA molecule, the MB undergoes a spontaneous conformational reorganization that forces the stem apart, leading to the restoration of fluorescence (ON state). Due to the inherent fluorescence signal transduction mechanism, an MB functions as a sensitive probe with a high signal-to-background ratio for real-time monitoring. Therefore, molecular beacons have a significant advantage over other fluorescent DNA probes in ultrasensitive analyses and have been providing a variety of exciting opportunities in DNA, RNA, and protein studies.^{3–8}

Molecular beacons can also be used in situations where it is not possible or desirable to isolate the probe–target hybrids from an excess of the unhybridized probes, for example, in the real-time monitoring of polymerase chain reactions in sealed tubes or in the detection of mRNAs within living cells. A novel method based on MB was developed to assay single-stranded DNA cleavage by single-strand-specific nucleases.⁸ This method permits real-time detection of DNA cleavage and makes it easy to characterize the activity of DNA nucleases and to study the steady-state cleavage kinetics. Therefore, an understanding of how a molecular beacon interacts with the target DNA is of great theoretical and practical importance.

Atomic force microscopy (AFM) is a versatile tool for imaging the surface structure of individual molecules and molecular assemblies in a wide variety of biological specimens.^{9–11} Because it is possible to measure intermolecular forces as low as the piconewton range, AFM is becoming a rapidly developing technique for probing affinity and recognition properties at the molecular level.¹² Compared to other sensitive methods for force measurements, AFM has the advantage of high force resolution and high spatial resolution and the capability to operate under physiological conditions for investigations of specific interactions in biological processes, such as intermolecular hydrogen bonds,¹³ electrostatic interactions,¹⁴ ligand–receptor complexes,^{15,16} antigen–antibody complexes,^{17–18} aptamer–protein interaction,¹⁹ and nu-

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cleic acid hybridization processes.^{20–22} In these applications, one interacting partner is immobilized on the AFM cantilever tip, and the interaction with the other partner on the sample surface is probed with force–distance curves. When the cantilever is withdrawn from the surface, the rupture force, defined as the maximum force at the moment of detachment, is recorded and used as a measure of the interaction of the probe with the target DNA on the surface.

In this work, we studied the specific recognition capability of MB by measuring the rupture force between the MBs and their target DNA. Biotinylated MB was covalently linked to the AFM tips using the streptavidin–biotin complex bridge structure. The rupture force of the MB interaction with the target DNA (complementary and one-base mutation) immobilized on a gold substrate was measured and compared to the interaction force between a linear DNA probe and the target DNA. The results indicate that the specific discrimination capability of the MB is higher than that of the linear DNA probe. To our knowledge, this is the first demonstration of AFM force measurement to study the high specificity of the hairpin DNA hybridization process.

EXPERIMENTAL SECTION

Materials. Biotinylated MB (5′-biotin-CCTAGC GGG CGC ACC TCT CTT TAC GCTAGG-3′), biotinylated cDNA1 (5′-biotin-GTA AAGAGAGGTGCGCCC-3′, complement to the MB), biotinylated cDNA2, (5′-biotin-GTA AAGAGAGGTGAGCCC-3′) complement with single-base mismatch, biotinylated linear DNA probe, LDNA (5′-biotin-CCTAGC GGG CGC ACC TCT CTT TAC), thiol-modified hairpin DNA probe (5′-SH-TTTTTTTCCTAGC GGG CGC ACC TCT CTT TAC GCTAGG-3′), and two 5′-thiol-modified ssDNAs (5′-HS(CH₂)₆-GTA AAGAGAGGTGCGCCC-3′ and 5′-HS-(CH₂)₆-GTAAAGAGAGGTGAGCCC-3′, complement and single-base mismatch) were synthesized by Shanghai Shengong Biotechnology Co. (Shanghai, China) and used without further purification. Biotinylated bovine serum albumin (BSA-biotin) and affinity-purified streptavidin were purchased from Sigma (St. Louis, MO). All chemicals were analytical reagents and were used without further purification. Milli-Q purified water (18.2 MΩ) was used for all sample preparations.

Functionalization of AFM Tips. Commercial silicon nitride cantilevers (Seiko) with standard V-shape were functionalized with biotinylated MB according to the following procedure.²³ Si₃N₄ tips were immersed in acetone for 5 min and then irradiated with ultraviolet light for 30 min. The irradiated tips were incubated in 50 μL of 1 mg/mL biotinylated BSA solution in 100 mM NaHCO₃ overnight at 37 °C and then rinsed six times with phosphate buffer (PBS: 20 mM Na₂HPO₄, 150 mM NaCl, pH 7.2). Streptavidin was coupled to the tip during a 30-min incubation in 50 μL of streptavidin (1 mg/mL in PBS) at room temperature. After rinsing with PBS to remove unbound streptavidin molecules, the streptavidin-functionalized tip was immersed in 250 nM biotin-MB solution at 4 °C overnight. The modified tips were stored in 20 mM PBS at 4 °C for future use.

Target DNA Immobilization. Gold substrates were prepared by vacuum evaporation of high-purity gold (99.99%) onto the cleaned silicon (100) wafer that had been precoated with chromium to improve adhesion (typically 200 nm of Au, 10 nm of Cr). Gold substrates were cleaned with “pirahana” solution (98% H₂SO₄:H₂O₂ = 7:3, v/v) prior to use and rinsed with water. The cleaned gold surfaces were plasma etched, heated for 5 min at 150 °C, and washed in absolute ethanol for 15 min. The self-assembled monolayer of HS-ssDNA surfaces was formed by immersing the gold substrates in a 250 nM HS-ssDNA solution in 0.4 M phosphate buffer (pH 7.3) for 3 h. Before analysis or hybridization, each sample was rinsed thoroughly with deionized water. The self-assembly and hybridization processes were monitored by surface plasma resonance²⁴ (SPR). A self-made SPR spectrometer of the Kretschmann configuration employing a tungsten halogen lamp was used. Details of the apparatus have been described previously.²⁵ The sensing mechanism of this SPR instrument utilized a fixed angle of incidence and modulated the wavelength. Wavelength resolution of the system was 0.2 nm.

AFM Force Measurements and Data Analysis. All force measurements were performed with an SPA400 atomic force microscope (Seiko Instrument) equipped with a piezoscanner having a maximum scan range of 20 μm × 20 μm × 2 μm. All experiments were carried out in a liquid cell filled with freshly prepared hybridization buffer (20 mM Tris-HCl, 0.1 M MgCl₂, pH 8.0) at room temperature. The system employs commercial cantilevers, which were modified as described above. The force constants of the cantilevers were not calibrated. Force measurements were obtained between biotinylated MB-functionalized tips and thiolated target DNA self-assembled gold substrates, between biotinylated linear DNA probe modified tips and thiolated target DNA self-assembled gold substrates, and between biotinylated target DNA modified tips and thiolated MB immobilized substrates. All force–distance curve measurements were performed when the tip was not being scanned in the lateral direction. The loading rate of the force–distance measurements ranged from 43 to 2900 nm/s. Taking into account the statistical nature of a single interaction event, many hundreds of approach–retract cycles were carried out with a single modified tip. To avoid misinterpretation due to arbitrary choice of “good” or “bad” curves, all measurements were included in the averaging process. The mean rupture force of molecules involved in the unbinding event was obtained, and the uncertainty as the 95% confidence limits was calculated.

RESULTS AND DISCUSSION

Surface Modification. Surface modification of the AFM cantilever tip and substrate is a key step in the successful investigation of the specific interactions of biomolecules by AFM force measurements. In an ideal immobilization strategy, the biomolecules should be tightly and irreversibly immobilized and also oriented properly for specific recognition. In this study, the AFM tip modification was achieved by covalently linking biotinylated MB to tips based on the biotin–streptavidin interaction using the bridge immobilization method,²⁶ which was easily adapted for

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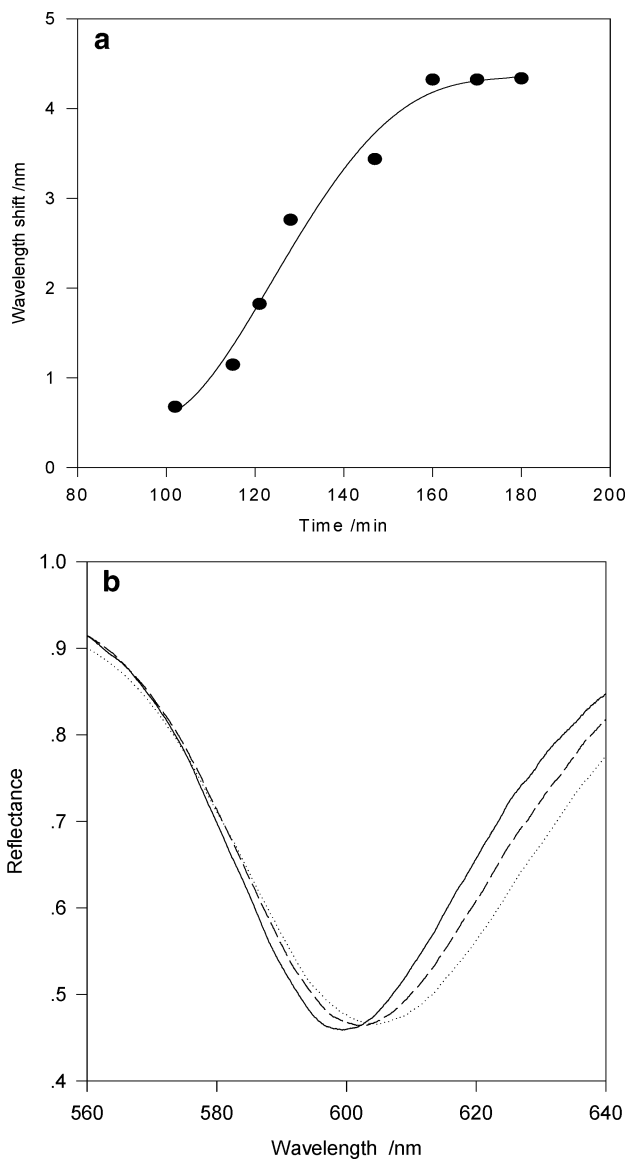


Figure 1. Response signal of the self-assembly process and hybridization of thiolated DNA on the gold substrates measured by SPR. (a) Self-assembly of 250 nM thiolated DNA on the gold substrate in 0.4 M phosphate, pH 8.0. After 3 h, the SPR response has reached a plateau. (b) Hybridization of the immobilized thiolated DNA with free MB. Solid line, baseline signal; dashed line, signal after self-assembly of thiolated DNA on the gold substrate; dotted line, signal after hybridization of self-assembled thiolated DNA with free MB.

this purpose. The essentially irreversible and highly specific biotin/avidin and biotin/streptavidin interactions are prototypical systems for ligand–receptor studies and have been utilized in a variety of analytical methods.²⁷ After preparing the surfaces by adsorption of BSA with attached biotin, the biotin was linked to

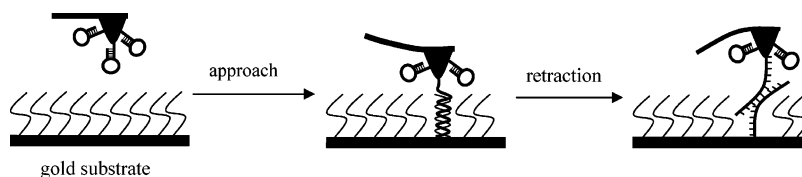


Figure 2. Schematic representation of the interaction between biotinylated MBs, modified AFM tip, and thiolated DNA-coated gold substrate (not drawn to scale). As the tip is brought in contact with the surface, MBs and target DNAs are brought into close contact, and the MB hairpin opens to form the DNA duplexes.

streptavidin, which was subsequently linked to the biotinylated MB. Beebe and co-workers have characterized the immobilization of protein onto the tip.²⁸ The high affinity of biotin–streptavidin complexes immobilizes the biotinylated MB tightly on the tips, and the bridge method enhances the motional freedom of the immobilized MB and the hybridization efficiency for force measurements.

Target DNA was immobilized on the substrates via thiolated DNA self-assembly on the gold surface. Surface coverage of immobilized ssDNA greatly affects the DNA hybridization efficiency. The self-assembly process of SH-ssDNA was studied by SPR, an optical technique used to quantify changes in mass at an interface. The sensing mechanism of this SPR instrument utilized a fixed angle of incidence and modulated the wavelength. Intermolecular interactions on the film surface cause a change in the refractive index of interface between the gold film and the solution, which induces a shift of resonance wavelength. As shown in Figure 1a, the wavelength leveled off after ~160 min, indicating that the self-assembly of SH-ssDNA was completely accomplished within 3 h. The hybridization between immobilized SH-ssDNA and free biotinylated MB was also monitored by SPR, as shown in Figure 1b. An obvious wavelength shift indicated that thiolated DNA had not only been immobilized but had also been hybridized with biotinylated MB.

Specific Interaction between MB and Target DNA. The force measurement for one of the many interactions between MBs and target DNAs is illustrated in Figure 2. As the tip approaches the DNA-coated gold substrate, the MB, and the target DNA are brought into close contact, and the MB hairpin opens to form the DNA duplex. In the experiments, the rupture force needed to separate the two strands is measured as a function of the distance between the tip and the gold substrate. The maximum cantilever deflection during the retraction phase is related to the magnitude of the force required to break the DNA duplex. A representative force–distance curve, shown in Figure 3A, indicates that significant adhesion existed in the MB–target DNA system. A histogram of rupture forces for one set of measurements with the same tip is shown in Figure 3B and Figure 3C. There is a spread of force values because of the variation of tip–surface contact area for different spots, the thermal fluctuations of AFM instrument, and the distribution of cDNA on the substrate. The mean rupture force of the MB–target DNA hybrid at a given loading rate was determined from force data of more than 100 measurements using a single tip and the same experimental conditions. The results in Table 1 show that a clear difference existed between MB/cDNA1 and MB/cDNA2 interactions, indicating that AFM can be used to detect a single-base mutation. All rupture forces detected in this experiment represent the total force of all contributing interactions. The rupture force for a single MB/

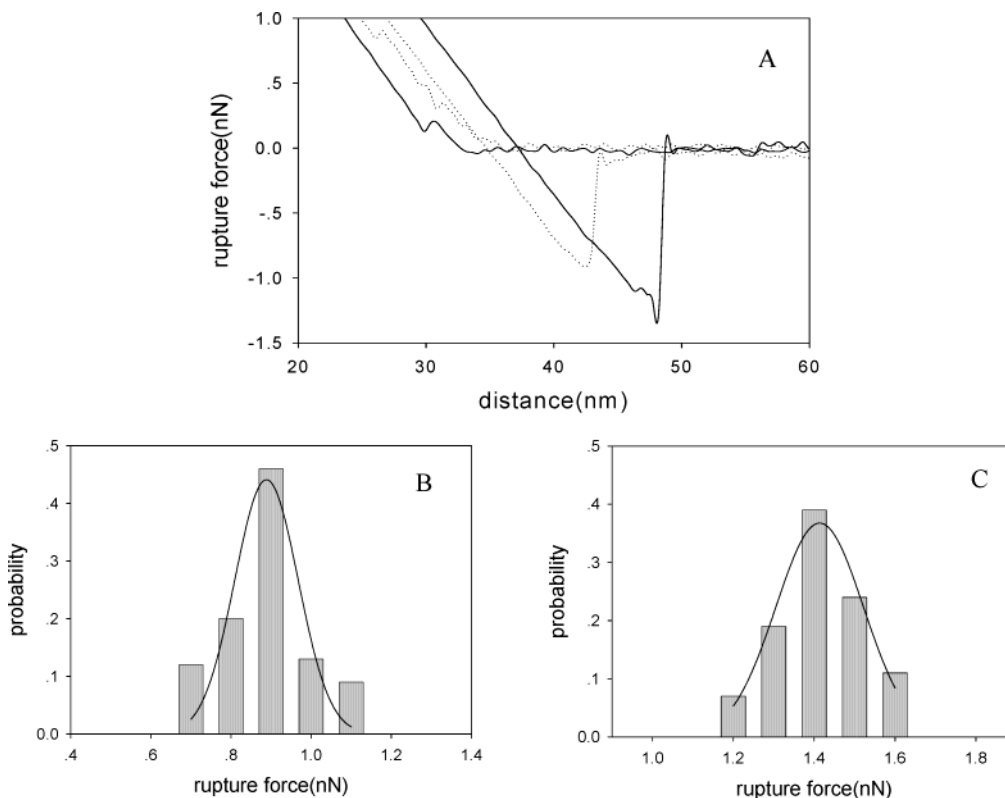


Figure 3. Typical force–distance curves and rupture force distribution histograms for MB with the target DNA. (A) approach–retraction cycle of force–distance curves of MB–cDNA1 (solid line) and MB–cDNA2 (dotted line) in hybridization buffer at pH 8.0. (B) Rupture force histogram corresponding to the force distribution of MB-modified tip and cDNA2-coated surface (average rupture force 0.87 ± 0.10 nN). (C) Rupture force histograms corresponding to the force distribution of MB-modified tip and cDNA1-immobilized surface (average rupture force 1.49 ± 0.13 nN). Each of the histograms was derived from 100–200 force measurements of adhesion events measured at different spots acquired with a single tip.

Table 1. Results (Mean and 95% Confidence Limits) of MB/Target DNA and LDNA/Target DNA Rupture Force Measurements^a

modified tip	modified substrate	rupture force, F (nN)	$\Delta F = \text{cDNA2} - \text{cDNA1}$ (nN)
MB ¹	cDNA1	1.50 ± 0.11	
MB ¹	cDNA2	0.92 ± 0.09	-0.58 ± 0.14
MB ²	cDNA1	1.76 ± 0.10	
MB ²	cDNA2	1.03 ± 0.13	-0.73 ± 0.16
MB ³	cDNA1	1.67 ± 0.11	
MB ³	cDNA2	0.86 ± 0.05	-0.81 ± 0.12
MB ⁴	cDNA1	1.36 ± 0.10	
MB ⁴	cDNA2	0.86 ± 0.04	-0.50 ± 0.11
MB ^a	cDNA1	1.46 ± 0.10	
MB ^b	cDNA1	1.45 ± 0.12	
LDNA ¹	cDNA1	0.96 ± 0.04	
LDNA ¹	cDNA2	0.82 ± 0.03	-0.14 ± 0.05
LDNA ²	cDNA1	1.19 ± 0.06	
LDNA ²	cDNA2	0.98 ± 0.05	-0.21 ± 0.08
LDNA ³	cDNA1	1.35 ± 0.06	
LDNA ³	cDNA2	1.23 ± 0.04	-0.12 ± 0.07

^a Numbers 1–4 represent different biotinylated MB or LDNA modified tips. (a) and (b) represent repeated experiments (b measured 4 days after a) with the same tip and substrates. All force measurements were detected in hybridization buffer (20 mM Tris-HCl, 0.1 M MgCl₂, pH 8.0) at room temperature. The uncalibrated spring constant of the cantilevers was 0.08 N/m.

DNA interaction was calculated using a Poisson statistical method^{30,31} for the two target DNAs (data not shown), and the results were the same order of magnitude as those reported by other groups.²⁹ Since the goal of this work was to compare the hybridization specificity of MB and LDNA with the target cDNAs,

and not to obtain single-molecule force values, the mean rupture force of all molecules involved in the unbinding event was used.

Replicate rupture force measurements were performed with the same tip after 4 days (MB^a and MB^b in Table 1), and the average values were clearly within experimental error. Control experiments were also performed to show that MB/target DNA force curves originated from specific MB/target DNA hybridization interactions. The reverse configuration, with target DNA on the AFM cantilever tip and MB on the substrate, was studied, and similar results were obtained (data not shown). It was also important to verify the absence of binding events at positions on the surface where no target DNA molecules were present. The rupture force between the MB-functionalized tip and the bare gold substrate was found to be 0.23 ± 0.07 nN, which is much lower than that between MB-functionalized tip and target DNA-coated substrate. In the MB/cDNA1 (MB modified on the tip) and cDNA1/MB (cDNA1 modified on the tip) systems, however, a

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clear force hysteresis was present as a result of the adhesion force between the MB–cDNA1 and cDNA1–MB pairs. This suggested that the force measured in the MB–cDNA system can be attributed to the specific interaction between MB and target DNA.

Comparison of MB/Target DNA and the LDNA/Target DNA Interactions. To better understand the general principles of the interaction with target DNA, we investigated the difference of rupture forces between MB/target DNA and linear DNA/target DNA. Biotinylated linear DNA probe was coated on the AFM cantilever tips, and the thiolated target DNA was immobilized using the same method as mentioned above. The force–distance curves for the LDNA–target DNA were obtained with the same experimental conditions used with the MB–target DNA. As summarized in Table 1, the change in the mean rupture force for linear DNA interactions with the two target DNAs (complementary and one-base mismatch) is considerably less than the change when MB is used. For LDNA/cDNA1 and LDNA/cDNA2, the maximum force difference was only $(0.21 \pm 0.08 \text{ nN})$, which is clearly lower than the minimum difference of $(0.50 \pm 0.11 \text{ nN})$ between MB/cDNA1 and MB/cDNA2. Because of its hairpin structure, the molecular beacon shows a large decrease in the extent of hybridization to the one-base mutated DNA due to spatial resistance. These results clearly show that MB has a higher specificity than linear DNA for monitoring target DNA having a base mutation.

To demonstrate the reliability of the experimental results, we repeated experiments with different tips and the substrates, and the results are shown in Table 1. These were carried out using different spots on the same surface and with several different surfaces. The distribution of adhesion forces may be attributed to uncalibrated tip spring constants, thermal fluctuations, and different molecular configurations. However, the major causes are probably variations in substrate pretreatment, tip–surface contact area, and sample immobilization. Because the sample immobilization process is crucial to the reliability of the force measurements, better sample treatment methods should be investigated. We have also tried to measure single-molecule forces. The single-molecule force range is $\sim 90 \text{ pN}$ (Supporting Information). These results

further confirm our measurements in the comparison study of the hybridization properties of MBs and linear DNA.

CONCLUSION

For the first time, the specific interactions between MB and target DNA have been successfully studied by atomic force microscopy. The hybridization specificities of both a molecular beacon and a linear DNA probe have been investigated by mean rupture force measurements. The rupture force for the MB/cDNA2 interaction was less than that for MB/cDNA1 by at least $0.50 \pm 0.11 \text{ nN}$, but when linear DNA was used, the difference was at most $0.21 \pm 0.08 \text{ nN}$. These results show that the MB is much more specific than linear DNA to base mismatches in the target. Several control experiments with different tips and substrates were performed to ensure the reliability of the method, and consistent results were obtained. Our results confirmed that the rupture force measured in this experiment is the specific interaction force between molecular probes and target DNAs.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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