J|A|C|S
ARTICLES
Published on Web 10/07/2004

Rupture Force between the Third Strand and the Double Strand within a Triplex DNA

Liansheng Ling, Hans-Jürgen Butt, and Rüdiger Berger*

Contribution from the Max-Planck Institute for Polymer Research, Mainz 55128, Germany

Received June 21, 2004; E-mail: berger@mpip-mainz.mpg.de

Abstract: The rupture force to separate the third strand and the duplex within a triplex DNA was measured by means of atomic force spectroscopy. The tip and the sample surfaces were functionalized by oligodeoxyribonucleotides 5′-TTCTTCTTTCTTTTCCTTTTCTTTCTTCTTACTTCTCTCTCTC TCTCTCT-SH-3′. The sample surface was hybridized with 5′-AAGAAGAAAGAAAAGGAAAAGAAAGAAGAA-3′ to form a double strand DNA on the surface prior to the force measurements. These sequences form triple helices with 30 base pairs under a pH of 5.8 and in the presence of 2.0 mM spermine. Signals of rupture of single and multiple triplex DNA were observed in the force distance curves. Rupture force histograms revealed a force of 42.6 ± 1.9 pN from 24 independent measurements at a tip velocity of 400 nm/s to separate the third strand from duplex DNA. The velocity dependence of the rupture force quantum indicates a thermal dissociation process similar to that of rupturing a ds-DNA. The number of rupture events was controlled by adding oligonucleotides 5′-AAGAAGAAAGAAAAGGAAAAGAAAGAAGAA-3′ either to reduce or to initiate triplex formation.

Introduction

Nucleic acid triplex structures were discovered in 1956 by Davies and Rich.[1] Since then, DNA triplexes have attracted considerable attention because of their importance in gene manipulation and gene therapy. Oligonucleotides generally bind to the major groove of the double stranded DNA (ds-DNA), where they specifically recognize oligopyrimidine−oligopurine regions by forming triple helices. These triplex forming oligonucleotides and their analogues can act as sequence specific inhibitors of translation (antisense reagents),[2] inhibit DNA transcription, replication, create site specific mutations, cleave DNA, and induce homologous recombination.[3] Therefore, better understanding of the stability of triplex DNA is important in curing gene related diseases and in controlling other biological activities, such as cancer. For that reason, interaction energy between Hoogsteen base pairs within triplex DNA has been studied theoretically.[4] It revealed that the energy of a Watson−Crick bond is a little bit higher than that of a Hoogsteen bond.

With atomic force microscope (AFM) spectroscopy, it is possible to measure specific interactions between individual molecules at small length scales and high sensitivity down to forces of a few piconewtons.[5] Hereby, the forces acting between a sharp tip and a flat surface are probed by measuring the deflection of a micromechanical cantilever spring. Specific interaction forces can be investigated by functionalizing the tip and the surface, respectively. This technique was first used in 1994 for force measurements in the study of the interaction forces between self-assembled monolayers terminated by CH_3 groups.[6] Afterward, the specific forces between avidin−streptavidin,[7] donor−acceptor,[8] aptamer−protein,[9] host−guest pairs,[10] and other molecular pairs[11] have been studied. The hydrogen bonds formed between Watson−Crick base pairs have been investigated with this technique in detail.[12] The latter experiments were realized by functionalizing the tip with single strand DNA (ss-DNA) at the 3′ (or 5′) end and by functionalizing the surface with its complementary strand at its 3′ end (or 5′). Hybridizations occur when the tip is brought close to the surface. During the subsequent withdrawal of the tip, the ds-DNA

(1) (a) Rich, A.; Davies, D. R. *J. Am. Chem. Soc.* **1956**, *78*, 3548−3549. (b) Felsenfeld, G.; Davies, D. R.; Rich, A. *J. Am. Chem. Soc.* **1957**, *79*, 2023−2024.
(2) Knudsen, H.; Nielsen, P. E. *Nucleic Acids Res.* **1996**, *24*, 494−500.
(3) (a) Vasquez, K. M.; Narayanan, L.; Glazer, P. M. *Science* **2000**, *290*, 530−533. (b) Maine, I. P.; Kodadek, T. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 1119−1124. (c) Radhakrishnan, I. R.; Patel, D. J. *J. Am. Chem. Soc.* **1993**, *115*, 1615−1617. (d) Hanss, B.; Bruggeman, L. A. *Curr. Opin. Nephrol. Hy.* **2003**, *12*, 439−445.
(4) Peters, M.; Rozas, I.; Alkorta, I.; Elguero, J. *J. Phys. Chem. B* **2003**, *107*, 323−330.
(5) Poggi, M. A.; Bottomley, L. A.; Lillehei, P. T. *Anal. Chem.* **2002**, *74*, 2851−2862.
(6) Frisbie, C. D.; Rozsnyai, L. F.; Noy, A.; Wrighton, M. S.; Lieber, C. M. *Science* **1994**, *265*, 2071−2074.
(7) (a) Moy, V. T.; Florin, E. L.; Gaub, H. E. *Science* **1994**, *266*, 257−259. (b) Florin, E. L.; Moy, V. T.; Gaub, H. E. *Science* **1994**, *264*, 415−417.
(8) Skulason, H.; Frisbie, C. D. *J. Am. Chem. Soc.* **2002**, *124*, 15125−15133.
(9) Jiang, Y. X.; Zhu, C. F.; Ling, L. S.; Wan, L. J.; Fang, X. H.; Bai, C. L. *Anal. Chem.* **2003**, *75*, 2112−2116.
(10) (a) Schönherr, H.; Beulen, M. W. J.; Bügler, J.; Huskens, J.; Van Veggel, F. C. J. M.; Reinhoudt, D. N.; Vancso, G. J. *J. Am. Chem. Soc.* **2000**, *122*, 4963−4967. (b) Tromas, C.; Rojo, J.; de la Fuente, J. M.; Barrientos, A. G.; Garcia, R.; Penades, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 3052−3055. (c) Zapotoczny, S.; Auletta, T.; de Jong, M. R.; Schönherr, H.; Huskens, J.; van Veggel, F. C. J. M.; Reinhoudt, D. N.; Vancso, G. J. *Langmuir* **2002**, *18*, 6988−6994. (d) Kado, S. Kimura, K. *J. Am. Chem. Soc.* **2003**, *125*, 4560−4564. (e) Auletta, T.; de Jong, M. R.; Mulder, A.; van Veggel, F. C. J. M.; Huskens, J.; Reinhoudt, D. N.; Zou, S.; Zapotoczny, S.; Schönherr, H.; Vancso, G. J.; Kuipers, L. *J. Am. Chem. Soc.* **2004**, *126*, 1577−1584.
(11) (a) Janshoff, A.; Neitzert, M.; Oberdörfer, Y.; Fuchs, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 3213−3237. (b) Samori, B. *Chem.−Eur. J.* **2000**, *6*, 4249−4255.
(12) Boland, T.; Ratner, B. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5297−5301.

10.1021/ja046341k CCC: $27.50

ruptures. The force at which rupture occurs is detected by measuring the deflection of the cantilever spring. Since the first report in 1994, the rupture force between two complementary single strand DNA has become an active research area.[13] The rupture force to separate both ss-DNA within a ds-DNA varies from 20 to 50 pN, depending on the number of base pairs within the ds-DNA and the velocity during withdrawal to rupture the ds-DNA.[14]

In another approach, the unzipping force of Watson−Crick base pairs in ds-DNA was studied by laterally moving the AFM tip,[15] by optical tweezers,[16] and by microneedle techniques.[17] The unzipping is realized by attaching the 3′ end of the ss-DNA to the tip and by attaching the 5′ end of its complementary strand to the surface. This allows one to measure the force to separate individual base pairs. A force of 10−15 pN is necessary to open ds-DNA. An unbinding force of 20 ± 3 pN for G-C base pairs and of 9 ± 3 pN for A-T base pairs has been revealed.

Two main different kinds of triplex DNA have been reported. One is a parallel triplex DNA, which contains C·G-C and T·A-T triplets, and the other one is an antiparallel triplex DNA, which contains G-G·C, A-A·T and T-A·T triplets (· represents the Watson−Crick hydrogen bond and - represents the Hoogsteen hydrogen bond). It was found that at acidic conditions the triplex DNA with a sequence rich in C·G-C triplets is more stable than triplex DNA having the same length but containing solely T·A-T triplets.[18] At neutral pH, cytosine is partly deprotonated and C·G-C triplets exert a destabilizing effect on the triplex DNA. Therefore, as long as cytosine is contained in the third strand, acidic conditions are necessary for the formation of triplex DNA. Another important condition for triplex formation at room temperature is the presence of cations.[18,19]

To investigate the stability of triplex DNA, we used the AFM spectroscopy technique to measure the rupture force between the third strand and the double strand within a triplex DNA.

Experimental Section

Design of Oligonucleotides. Oligonucleotides with a length of 50 bases have been selected. Within this ss-DNA, 30 homopyrimidine bases are used for triplex formation. The additional 20 bases act as a spacer between the part that hybridizes and the surface. This allows one to separate the rupture force from the adhesion force signals between the tip and the surface because rupture events occur a few nanometers away from the surface. To bind ss-DNA covalently to surfaces, mercapto modified oligodeoxyribonucleotide with a sequence of 5′-TTCTTCTTTCTTTTCCTTTTCTTTCTTCTTACTTCTCTCTCTCTCTCTCT-SH-3′ (oligo A) are used. Oligonucleotides with a sequence of 5′-AAGAAGAAAGAAAAGGAAAAGAAAGAAGAA-3′ (oligo B) are used to form a ds-DNA with oligo A. Oligo A and oligo B from the surface together with oligo A from the tip form triple helical structures (Figure 1). Hereby, only the underlined bases are designed for triplex DNA formation. For control experiments, ds-DNA is formed using the oligonucleotides 5′-AAGAAGAAAGAAAAGGAAAAGAAAGAAGAACTCTCTCTCTCTCTCTCTCT-SH-3′ (oligo C). All the oligonucleotides were purchased from MWG (MWG Biotech, D-85560 Ebersberg, Germany). High pressure liquid chromatography (HPLC) was used to purify the oligos and mass spectrometry was performed to verify the mass.

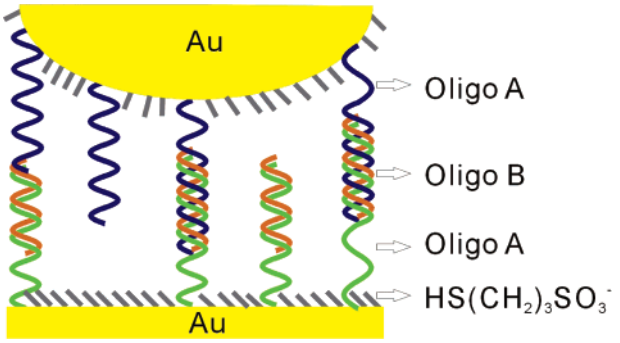

Figure 1. Scheme of tip and sample interface for measuring rupture forces with the atomic force microscope spectroscopy technique. The AFM tip is functionalized with oligo A (dark blue), which forms a triplex with the ds-DNA functionalized surface of oligo A (green) and oligo B (orange). Triplex DNA is formed, even with molecules not situated at the tip apex, owing to the previous contact of tip and surface. The area of contact depends on the maximal loading force of the tip against the surface. For a maximal force of typically 1 nN and a tip radius of 70 nm, a contact area of 3 nm^2 is calculated from the Hertz model.[25] In addition, the flexible DNA layer of 21 nm thickness has to be considered, as well as a ring around the tip contact area. This increases the contact area to an upper limit of 1500 nm^2. In addition, some ss-DNA or ds-DNA do not form a triplex, owing to a missing counterpart. During the withdrawal of the tip from the surface, oligonucleoteides might be stretched before the triplex helices rupture (outlined on the right-hand side).

Sample Preparation. Gold (200 nm) was deposited on freshly cleaved mica by thermal evaporation or sputtering (BALTEC MED 020, BALTEC, 58454 Witten/Buhr, Germany). Then, the gold surface was adhered on a metal substrate by means of a two component epoxy glue; subsequently, the mica was stripped, which results in a smooth gold surface. Typically, we obtained a root mean square roughness of 0.4 nm on an area of 1 μm^2. Prior to functionalization with oligonucleotides, the gold surface was exposed for 300 s to an air−plasma for cleaning. We fabricated a special Teflon container that has a reservoir with a diameter of 6 mm and a depth of 2.5 mm and has holes for injection and waste of liquids. The gold surface was placed on top of the reservoir and sealed by an O-ring. Then, a 4.0 μM aqueous solution of oligo A was injected into the reservoir (volume: 137 μL) by a syringe. After a 20 min immobilization, 140 μL of 1.2 μM 3-mercapto-1-propanesulfonic sodium ($HS(CH_2)_3SO_3Na$, purity: 90%, as obtained from Aldrich Co) was injected into the container to remove the oligonucleotide solutions and to passivate the uncovered gold surface by a monolayer of $HS(CH_2)_3SO_3Na$ for about 15 h. Alkanethiols can replace other self-assembled monolayers of alkanethiols[20] within hours and days. In our case, part of the

(13) (a) Lee, G. U.; Chrisey, L. A.; Colton, R. J. *Science* **1994**, *266*, 771−773. (b) Noy, A.; Vezenov, D. V.; Kayyem, J. F.; Meade, T. J.; Lieber, C. M. *Chem. Biology* **1997**, *4*, 519−527. (c) Bustamante, C.; Smith, S. B.; Liphardt, J.; Smith, D. *Curr. Opin. Struct. Biol.* **2000**, *10*, 279−285. (d) MacKerell Jr., A. D.; Lee, G. U. *Eur. Biophys. J.* **1999**, *28*, 415−426.
(14) Strunz, T.; Oroszlan, K.; Schäfer, R.; Güntherodt, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11277−11282.
(15) (a) Rief, M.; Clausen-Schaumann, H.; Gaub, H. E. *Nature Struct. Biology* **1999**, *6*, 346−349. (b) Cocco, S.; Monasson, R.; Marko, J. F. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8608−8613. (c) Krautbauer, R.; Rief, M.; Gaub, H. E. *Nanoletters* **2003**, *4*, 493−496.
(16) (a) Essevaz-Roulet, B.; Bockelmann, U.; Heslot, F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11935−11940. (b) Bockelmann, U.; Thomen, Ph.; Essevaz-Roulet, B.; Viasnoff, V.; Heslot, F. *Biophys. J.* **2002**, *82*, 1537−1553.
(17) (a) Bockelmann, U.; Essevaz-Roulet, B.; Heslot, F. *Phys. Rev. Lett.* **1997**, *79*, 4489−4492. (b) Bockelmann, U.; Essevaz-Roulet, B.; Heslot, F. *Phys. Rev. E* **1998**, *58*, 2386-2394.
(18) (a) Faria, M.; Giovannangeli, C. *J. Gene Med.* **2001**, *3*, 299−310. (b) James, P. L.; Brown, T.; Fox, K. R. *Nucleic Acids Res.* **2003**, *31*, 5598−5606.
(19) (a) Leitner, D.; Schroder, W.; Weisz, K. *Biochemistry* **2000**, *39*, 5886−5892. (b) Wu, P.; Kawamoto, Y.; Hara, H.; Sugimoto, N. *J. Inorg. Biochem.* **2002**, *91*, 277−285.

bound DNA of the surface and the tip was replaced by 3-mercapto-1-propane-sulfonic sodium. This passivation of the surface was found to be essential because unsaturated surfaces would cause strong nonspecific adhesion of molecules.[15c] At higher concentrations, 1.5 μM of $HS(CH_2)_3SO_3Na$, and at a passivation time of 15 h, no rupture events have been observed. Therefore, a concentration of 1.2 μM was used throughout all experiments. For hybridization with oligo A, the complementary homopurine strands (oligo B, concentration 4 μM, volume: 140 μL) were injected into the container. After 10 min, the bis−tris buffer (pH 5.8, ion strength 50 mM, 2.0 mM spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), volume: 140 μL) was injected into the Teflon container. This washing process was used to remove physically adsorbed oligonucleotide molecules from the surface. The bis−tris buffer solution was used throughout all experiments to minimize contamination from other chemicals. Milli-Q water (18 MΩ) was used for preparing solutions and all experiments were carried out at room temperature (22−23 °C).

Tip Preparation. The tip sides of standard V-shaped silicon nitride cantilevers (MLCT-AUNM) with pyramidal tips (Veeco. Instr., Santa Barabara, CA) were subsequently coated with 2.0 nm Cr and 8.0 nm Au by thermal evaporation. Then, the cantilevers were immersed for 20 min in 4.0 μM oligo A aqueous solution. Afterward, the cantilevers were immersed in 1.2 μM of $HS(CH_2)_3SO_3Na$ aqueous solution for 15 h.

For the control experiment, we used oligo C instead of oligo A at the tip. All other parameters were identical. Oligo C forms a duplex helical structure with oligo A.

AFM Force Measurements and Data Analysis. All force measurements were performed with a MultiMode connected to a Nanoscope IIIa controller (Veeco Instr., Santa Barabara, CA). The setup was equipped with a PicoForce low noise head, which reduces cantilever sample interferences. A 10 μm scanner (type E) and a glass liquid cell were used for the experiments. For all experiments, cantilevers with a nominal spring constant of 0.01 N/m were selected. The tip radii were measured by means of scanning electron microscopy (SEM). Spring constants were independently measured by a thermal tune procedure described in the PicoForce manual. This method to estimate the spring constant usually has an error of 10−20%.[21]

After mounting the liquid cell and sealing it with an O-ring, the bis−tris buffer (pH 5.8) containing spermine was injected (this step should be carried out carefully to circumvent bubble creation and the liquid overflowing from the liquid cell). Spermidine, spermine, or mateal ions are used to stabilize triplex DNA.[22] Spermine can induce the formation of triplex DNA in the concentration range of 0.1 μM to 5 mM without inducing condensation.[22,23] Here, we selected 2.0 mM aqueous spermine solution as the triplex stabilizer. These environmental conditions are favorable for the formation of the triplex DNA. After fine adjusting the laser spot on the apex of the cantilever, the tip was brought toward the sample surface. Then, cantilever deflection versus distance curves were recorded at tip velocities between 20 and 2000 nm/s. A relative trigger of 60 nm

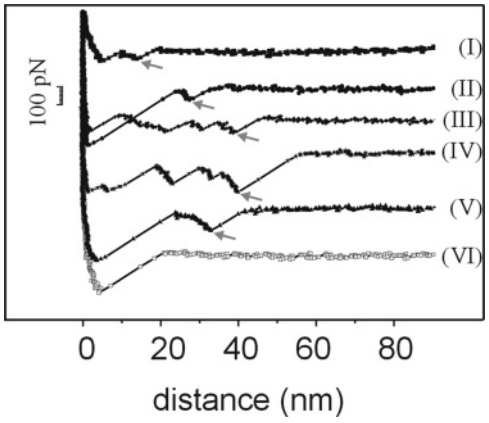

Figure 2. Six exemplary force distance curves recorded in aqueous bis−tris buffer (50 mM, pH 5.8, 2.0 mM spermine) between a tip functionalized with oligo A and a surface covered with ds-DNA of oligo A·oligo B. The cantilever used here has a spring constant of 14.3 mN/m, and the tip velocity was set to 400 nm/s. The radius of curvature of the tip was 62 nm. From the 1000 force distance curves, about 15% show clear rupture events.

deflection of the cantilever was used to control the maximal force (typically <1 nN) of the tip against the surface. From this deflection versus distance curve a force distance curve (FD) was obtained by multiplying the cantilever deflection with the spring constant of the cantilever and by subtracting the cantilever deflection from the height position. Usually, for each experimental condition, 1000 subsequent force curves were recorded at 10 different spots; 100 force curves were captured continuously at each spot. Data of rupture forces were plotted in histograms, which were subsequently subjected to Fast Fourier Transform (FFT) filtering and line fitting (Microcal Origin software) for analysis.[10c]

Results and Discussion

During the approach of the ss-DNA (oligo A) modified tip toward the ds-DNA (oligo A·oligo B), modified surface triplex DNA forms. These resulting bonds are ruptured during the subsequent withdrawal of the tip (Figure 1). The designed oligonucleotide sequences make sure that any shift of the third strand versus the ds-DNA induces, maximally, eight base triads matches for triplex formation in one period. This happens only for a shift of 22 base triads in the 3′ or 5′ direction to the homopurine strand. A triplex formed from only eight triads is not stable at room temperature of 22 °C.[24] Consequently, other sequences of bases in the designed oligonucleotide cannot form a triplex DNA under our experimental conditions. In other words, for the measurement of forces we either measure a complete rupture of one or more triplex DNA formed by all 30 base triads or none. In general, the number of formed triplex DNA is dependent on the radius of curvature of the tip, the maximal force loading of tip versus surface, the density of DNA on the tip and the sample surface, as well as the probability that a binding occurs.

Typical force distance curves that are selected from 1000 subsequent force curves using an oligo A modified tip and a ds-DNA modified sample surface are shown in Figure 2. Five out of the six curves exhibit signals that can be attributed to one or more rupture events of the formed triplexes (curves I−V). The last rupture event (LRE) of each force curve that occurs at

(20) Laibinis, P. E.; Fox, M. A.; Folkers, J. P.; Whitesides, G. M. *Langmuir* **1991**, *7*, 3167−3173.
(21) Levy, R.; Maaloum, M. *Nanotechnology* **2002**, *13*, 33−37.
(22) Soyfer, V. N.; Potaman, V. N. *Triple-Helical Nucleic Acids*; New York, 1996; p 194.
(23) (a) Lyamichev, V. *Nucleic Acids Res.* **1991**, *19*, 4491−4496. (b) Singleton, S. F.; Dervan, P. B. *Biochemistry* **1993**, *32*, 13171−13179.
(24) Singleton, S. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 6957−6965.

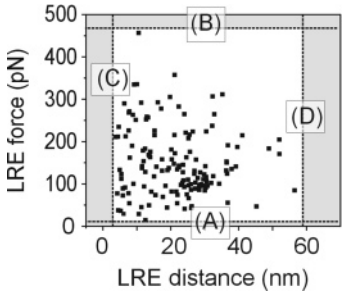

Figure 3. Correlation between forces of last rupture event (LRE) and its distances. Indicated are the boundary condition from noise that correspond to two times the standard deviation of 8 pN (A), the influence of DNA density and tip shape (B) as well as the overlapping effect arising from adhesion forces (C), and the capability to stretch the entire formed triplex ss-DNA entity (D).

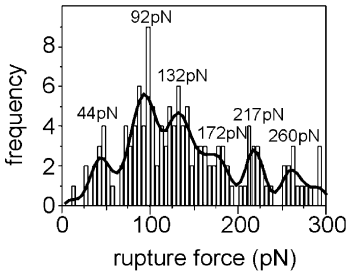

Figure 4. Histogram of LRE forces obtained from a set of 1000 force distance curves as shown in Figure 2. The solid lines represent the FFT-smoothed histograms. The peak values of the FFT analysis are written close to the peaks. All peak values can be used to fit the rupture force between the third strand and the double strand within a triplex DNA by a line.

the furthest distance from the surface is used for analysis. These events can be clearly attributed to a specific number of binding events. For other rupture events closer to the surface, the contribution of ruptured triplex DNA is not clear, owing to the influence of adhesion forces and/or a superposition of several rupture events at nearby distances (e.g., for both influences in curve IV). The arrows in Figure 2 indicate these LREs, respectively. The rupture force is taken as the force between the mean value of the baseline and the rupture force peak value. In the case of curve I, the rupture force corresponds to a value of 40 ± 8 pN. The LREs occur at different distances and rupture forces. The baseline at the right side at a distance larger than 60 nm indicates the noise level of a single measurement of approximately 8 pN standard deviation. For comparison, a FD curve not showing a rupture event is plotted (curve VI). All curves also display contact adhesion force at 0 nm distance. Rupture events could possibly have occurred between the tip and the surface within the adhesion peak. This contact adhesion also contains residual nonspecific interactions.

Oligo B is bound to oligo A by Watson−Crick bonds. The triplex between oligo A of the tip and ds-DNA (oligo A·oligo B) of the surface is formed by Hoogsteen bonds. The latter one is energetically less stable than that of Watson−Crick bonds. Therefore, the triplex is ruptured at the Hoogsteen bond or, in other words, the third strand is ruptured from the ds-DNA. Therefore, the probability that oligo B is transferred to the tip within the force distance curves is very low. This is supported by the time evolution of the rupture force measurement. Each measurement consists of 100 force distance curves of at least 10 different spots. We did not observe a decrease in the number of binding events, which would be a sign that oligo B is transferred to the tip.

To elucidate the role of the tip shape, the number of triplexes formed, and the mechanical properties of the ss-DNA and triplex DNA assembly, the LRE distance is plotted versus LRE force (Figure 3). No clear correlation is observed, only an accumulation of LRE has occurred at around 30 nm and at a LRE force around 100 pN. The length of a triplex DNA including the spacer DNA is calculated to be 32 nm.[26] In case the molecular entity would not stretch upon pulling, the last rupture events must occur at a distance of about 32 nm. From Figure 3, we see that there are LRE at distances larger than 40 nm. From this, we conclude that stretching of the triplex and/or ss-DNA occurs before it ruptures. Figure 3 can be used to visualize the boundary conditions and limits for that experiment. The lowest force measurable is given by the noise of the baseline (A) at 16 pN. The strongest possible force is given by the radius of curvature and the density of functional groups either on the tip or on the surface. This determines the maximal number of binding events between the tip and the surface, and thus the upper force limit (B). In other words, the larger the contact area or the higher the density of functional groups the more binding events occur, and the probability increases to observe larger forces for simultaneously ruptured triplexes. The lower limit for the LRE distance is determined by the contact adhesion force (C). The upper limit for the LRE distance is given by the mechanical properties of DNA (D).

The histogram of forces attributed to LRE between the ss-DNA modified tip and ds-DNA modified gold surface is presented in Figure 4. The peak positions were determined by filtering data with FFT smoothing.[10c] We attribute the peak values to a single rupture event (44 pN) and to multiple rupture events: 92 pN for two, 132 pN for three, 172pN for four, 217 pN for five, and 260 pN for six. For analysis, the rupture force peak values are plotted against the peak number. The slope of a fitted line going through the zero point was taken as the rupture force between the third strand and the double strand within a triplex DNA. This resulted in a rupture force for a single binding event of 43.1 ± 0.5 pN.

The experiment was repeated 24 times using different tips and samples to exclude effects from tip shape, spring constants, gold coating of the tip, and density variation of DNA molecules at the tip or surface. From all the experiments that have been carried out, the average value of the rupture force quantum is 42.6 pN with a standard deviation of 1.9 pN at a rupture velocity of 400 nm/s.

It is well known that the third strand binds in the major groove of ds-DNA with Hoogsteen bonds, while ds-DNA formation is based on Watson−Crick bonds. To compare the strength of the two kinds of bonds and to exclude experimental artifacts, we measured the rupture force for ds-DNA. This experiment is based on the same base sequence of DNA as used before for

(25) Hertz, H. *J. Reine Angew. Math.* **1881**, *92*, 156−171.

(26) This value is calculated from 5.6 Å × 20 × 2 + 3.16 Å × 30 = 318.8 Å; here, 5.6 Å is the length of a base in a single strand DNA [Williams, M. C.; Wenner, J. R.; Rouzina, I.; Bloomfield, V. A. *Biophys. J.* **2001**, *80*, 1932−1939] and 20 is the number of bases in the spacer of both strands. 3.16 Å is the length of one base triad in a triplex DNA [Asensio, J. L.; Carr, R.; Brown, T.; Lane, A. N. *J. Am. Chem. Soc.* **1999**, *121*, 11063−11070] and 30 represents the number of base triads in triplex DNA used here.

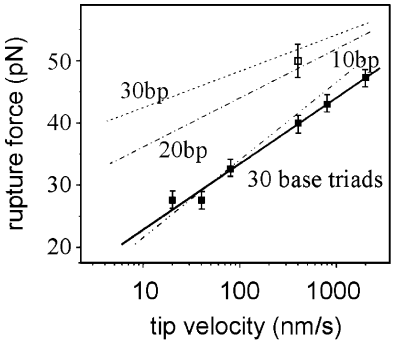

Figure 5. Dependence of the rupture forces of single triplex DNA with 30 base triads on tip velocity (filled squares). Each point is obtained from a set of 1000 force curves (range 200 nm at frequencies between 0.2 and 5.0 Hz) and a subsequent analysis as depicted before. The points at 40 and 20 nm/s are obtained from 400 force curves at a range of 100 nm. At a velocity of 20 nm/s, one force curve for a distance of 200 nm would require 20 s. A reduction of the range from a 200 to a 100 nm distance halves the measurement time and reduces errors arising from piezo and thermal drifts. For the same reason, we also reduced the number of force distance curves from 1000 to 400 at 20 and 40 nm/s velocities. In addition, the rupture force for a 30 base pair ds-DNA at the tip velocity of 400 nm/s is shown (open square). Here, the error bar reflects the measurement error including the spring constant. For comparison, the tip velocity dependence for ds-DNA with 30 bp (dashed line), 20 bp (dash−dotted), and 10 bp (dash−double-dotted), as taken from Strunz et al.,[14] are plotted.

the investigation of triplex DNA. Oligo A was bound to the tip and the surface was coated with oligo C. Between both ss-DNA a duplex DNA with a length of 30 base pairs can form during the tip approach. Under the same condition for rupturing triplex DNA, we found a force of 50.7 ± 2.5 pN for rupturing the ds-DNA at 400 nm/s. This value is in agreement with the value reported by Strunz et al.[14] In addition, these authors found that the rupture force for ds-DNA varies logarithmically with the tip velocity. This dependence was attributed to cooperative thermal dissociation of short oligonuceotides. We found that the rupture force quantum between the third strand and the double strand within a triplex DNA increased logarithmically with increasing tip velocity (Figure 5). For comparison, we plotted the velocity dependence of ds-DNA rupture forces for different ds-DNA lengths.[14] The velocity dependence of triplex DNA is similar to that of ds-DNA and can be understood as a system far from equilibrium.[27] This result suggests that cooperative thermal dissociation of short oligonuceotides also plays a crucial role in rupturing the triplex DNA. However, the rupture force for triplex DNA for 30 base triads is smaller than that for a rupturing ds-DNA having 30 base pairs. This is in agreement with ultraviolet spectroscopy data presented by Choi et al.,[28] which indicates that the stability of triplex DNA is smaller than that of ds-DNA.

The number of binding events for triplex formation can be controlled by the amount of complementary strands, which are able to hybridize upon contact of tip and surface. A complementary strand that is added to the solution could hybridize with oligos on the surface or the tip, depending on the specific sequence. This reduces the number of possible binding sites between the tip and the surface. As a consequence, the number

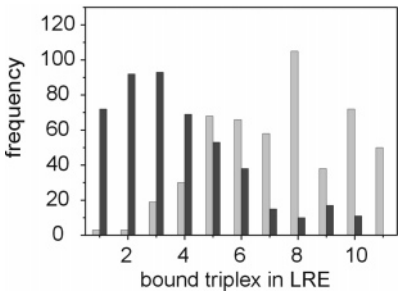

Figure 6. Histogram of the number of ruptured triplex DNA. The light gray histogram reflects the result recorded in pure buffer and the dark gray histogram for a concentration of 0.32 nmol of complementary homopurine strand oligo B in the buffer solution.

and type of LRE could therefore be a good measure for the presence and concentration of complementary strands in the vicinity of the tip and the surface. This was indeed observed in an experiment with oligo A on the tip and oligo A·oligo B ds-DNA on the surface when adding oligo B into the solution (a volume of 80 μL at a concentration of 4.0 μM, leading to an amount of 0.32 nmol). The peak value of the histogram of the number of molecules that were ruptured in the LRE shifted from 8 to a value of 2−3 (Figure 6). A significant drop in the total number of LRE from 3780 to 1718 was observed. This demonstrated that the presence of oligos can be detected by the rupture force method. In addition, the number of binding events can be controlled by the amount of complementary ss-DNA.

In contrast to the reduction of the number of hybridization events, the triplex formation allows one to detect the onset of a triplex hybridization reaction. In this case, a ss-DNA is detected in the solution, which can hybridize with a ss-DNA immobilized on one surface (tip or surface, Figure 7, parts a and b) by Watson−Crick bonds. As a consequence of these hybridizations, triplex binding may occur between the ds-DNA and ss-DNA when the tip approaches the surface (Figure 7c). The triplex is formed by Hoogsteen bonds between the ss-DNA and the ds-DNA. Such triplex DNA formations are then detected by rupture events in force distance curves. When the tip is withdrawn from the surface, triplex DNA ruptures at the Hoogsteen bonds. In other words, the rupture events happen between the third strand and the ds-DNA. Further increasing the concentration of ss-DNA in the solution leads to an increasing amount of ds-DNA formed on the tip and the surface. Consequently, no triplex can form during a force distance curve and the number of rupture events is decreased (Figure 7d).

We realized this experiment by functionalizing both the tip and surface with oligo A. Oligo A does not hybridize with itself (histogram I of Figure 7e). For a total number of 1000 force curves, we have detected only 17 signals similar to signals attributed to rupture events of triplex DNA. This background is attributed to contamination of the liquid cells from previous experiments and can be regarded as background for all experiments shown here. Then, the homopurine strand (oligo B), which is necessary for the formation of triplex DNA, was injected into the liquid cell, and the amount was increased step by step from 0.16, 0.32, 0.48, 0.64, and 0.72 nmol oligo B, respectively. The corresponding histograms of LRE induced by increasing oligo B concentration are presented in Figure 7e. Significant changes in the histograms are observed. After adding oligo B at an amount of 0.16 nmol, two peaks appear at 42 and 80 pN. These

(27) (a) Rief, M.; Gautel, M.; Oesterhelt, F.; Fernandez, J. M.; Gaub, H. E. *Science* **1997**, *276*, 1109−1112. (b) Marszalek, P. E.; Lu, H.; Li, H. B.; Carrion-Vazquez, M.; Oberhauser, A. F.; Schulten, K.; Fernandez, J. M. *Nature* **1999**, *402*, 100−103.

(28) Choi, S.-D.; Kim, M.-S.; Kim, S. K. Lincoln, P.; Tuite, E. Norden, B. *Biochemistry* **1997**, *36*, 214−223.

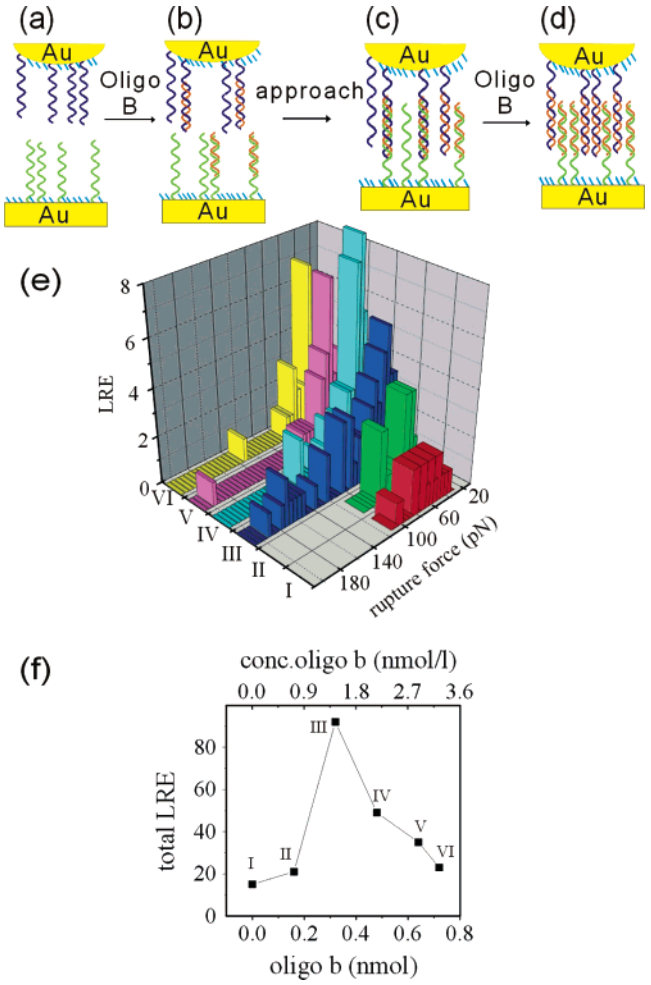

Figure 7. (a−d) Principle of an experiment to initiate triplex DNA formation between oligo A (dark blue = oligo A on the tip; green = oligo A on the surface) by adding the complementary homopurine strand oligo b (orange). (e) Histograms that result from sets of 1000 FD curves, each of which is recorded in a stepwise increasing amount of oligo B (curves I−VI). (f) The dependence of total LRE for the histograms shown in (e). All experiments are recorded in 2.0 mM spermine, 50 mM bis−tris buffer, pH 5.8, and at a tip velocity of 400 nm/s.

peaks remain while increasing the amount of oligo B to 0.54 nmol (II → IV). A maximum is reached for a concentration of 0.48 nmol oligo B. Further increasing of the concentration of oligo B results in a disappearance of the peak at 80 pN. Only the peak at about 40 pN is still there (V → VI).

In addition, the total number of LRE can be analyzed (Figure 7f). This value reflects the number of triplex DNA that have been formed and takes into account that several triplex bindings are ruptured at LRE. The total LREs increase with increasing amount of oligo B (I → II → III). After adding an amount of 0.36 nmol oligo B, the total number of LRE reached a maximum. Such a peak value of total LRE can be explained by an ideal matching condition of ss-DNA and ds-DNA on the tip and the surface. In other words, by adding this amount of oligo B, the possibility of triplex DNA formation reaches a maximum. With a further increase of the concentration of oligo B, more ds-DNA are present at the tip and the surface, which is unfavorable for the formation of triplex DNA (Figure 7d). This is reflected by a decrease of the total LRE (IV → VI in Figure 7f) close to the starting value where no oligo B is present (I).

Summary and Conclusion

The force for rupturing the third strand and the double strand within a triplex DNA with 30 triads is 42.6 ± 1.9 pN at a tip velocity of 400 nm/s. By considering the error owing to the estimation of the spring constant, the force quantum is within 42.6 ± 8 pN. In particular, this error considers a possible systematic instrumental error arising from the determination of the spring constant value. The force quantum depends logarithmically on the tip velocity, which is similar to that of rupturing duplex DNA, and reveals that cooperative thermal dissociation of short oligonuceotides also exists in the process of rupturing triplex DNA. These results demonstrate that the stability of the triplex DNA is in the same order as ds-DNA in the presence of the stabilizer spermine. Experiments have demonstrated that the number of rupture events can be controlled by the presence of oligonucleotides in the buffer solution.

Acknowledgment. We acknowledge Michael Kappl for developing the data analysis software and for fruitful discussions. Uwe Rietzler is acknowledged for continuous support in preparing gold coated cantilevers.

JA046341K