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Simultaneous AFM Manipulation and Fluorescence Imaging of Single DNA Strands

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We report combined atomic force and far-field fluorescence microscopic experiments which allow the simultaneous atomic force manipulation and optical observation of individual dye-labeled DNA molecules. A detailed understanding of the binding properties of DNA to different transparent surfaces is prerequisite for these investigations. Atomic force spectroscopy and fluorescence microscopy of single DNA strands yielded detailed insight into two different types of DNA binding onto transparent polylysine-coated and silanized glass surfaces. We subsequently demonstrate how the different binding can be exploited to perform two types of nanomanipulation experiments: On polylysine, strong electrostatic interactions over the whole length of the DNA strand enable the writing of micrometer-sized patterns. By contrast, the strong pointwise attachment of DNA to silanized surfaces allows horizontal stretching of single DNA strands to lengths exceeding 1.6 times the contour length of the DNA strand. With this new approach it is possible to directly observe the rupture of the strongly bonded DNA strand.

Introduction

During the last decade, optical and atomic force spectroscopy have independently been developed as techniques for the investigation of single molecules. The observation of only one molecule at a time avoids averaging and thus removes the need for external synchronization commonly required in bulk spectroscopy. Both techniques have therefore revealed a wealth of hitherto unavailable information. Today, optical single-molecule detection at room temperature relies almost exclusively on fluorescence excitation and detection. Fluorophores are investigated with an optical microscope using a high-sensitivity point or multichannel detector.[1,2] In many of these experiments, the fluorophores serve as probes for the exact location of a target structure. By contrast, in force-spectroscopic experiments on single molecules the mechanical properties of the molecule itself are of interest. Depending on the relevant force regime, different methods are applied. These include the stretching of molecules with magnetic beads,[3,4] optical tweezers,[5–7] and the atomic force microscope (AFM).[8,9] Simultaneous optical and AFM experiments on single molecules hold promise for opening new horizons both for manipulation on a nanoscopic scale and understanding the interplay between an applied mechanical force and the optical properties of molecules. While the first combinations of AFM and optical microscopy were presented more than a decade ago,[10] solutions to the problem of how to correlate the recorded images are still being sought.[11] The natural solution to this problem is near-field optical microscopy,[12] which, however, compromises AFM resolution. In addition, the metallic tip coatings used can alter the optical properties of the sample.

Here we report the simultaneous AFM manipulation and optical imaging of single DNA molecules. DNA was chosen as a model polymer for several reasons. It is one of the polymers with the most thoroughly described mechanical behavior on the single-molecule level[5–9] and has recently attracted considerable attention as a building material for nanostructures.[13,14] In addition, DNA can be labeled with intercalating fluorophores.[15] Usage of these dyes is especially appealing, since their fluorescence quantum yield is strongly enhanced on binding to DNA, which can thus be imaged against a weakly fluorescing background. Until now, force-spectroscopic characterization of DNA has exclusively been done on Au surfaces.[8,9,16] However, for combined optical and force experiments, the utilization of transparent surfaces is necessary. Different methods have been designed to immobilize and align fluorescently labeled DNA on transparent, coated glass surfaces.[17,18] Our experiments make use of these binding techniques. Subsequent optical imaging experiments were then done on both of these surfaces using DNA labeled with the intercalating dye TO-PRO-3. The results of these experiments provide the basis for combining the mechanical manipulation of a single molecule by an AFM tip with simultaneous optical observation. Two different manipulation experiments were performed: on polylysine, we demonstrate that the specific, strong electrostatic surface fixation allows the controlled deposition of the DNA from the tip and the formation of micrometer-sized DNA structures. On the silanized surface, in contrast, the selective terminal attachment of DNA can be exploited to

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perform a new type of lateral force spectroscopy, by horizontally stretching a single DNA strand. This allows single-molecule stretching experiments to be performed in the focal plane of a microscope. Thereby, the stretching process can be visualized in great detail by using fluorescence microscopy. The latter experiments allow investigation of the binding properties of the fluorophore to the DNA under the mechanically induced phase transitions observed in DNA force curves. Most interestingly, the AFM tip can be employed to stretch DNA strands attached to a silanized surface until they rupture. To our knowledge, this is the first direct visualization of a single polymer-rupture event, which has previously only been monitored indirectly via the force response of an AFM tip. Our technique of simultaneous optical observation and AFM manipulation promises to afford exclusive access to the relevant mechanical properties of polymer chains during strand scission.

Results and Discussion

Atomic Force Spectroscopy

As a first step we demonstrate that the force spectra characteristic of DNA are also accessible on specific surfaces with transparent coatings. These experiments give insight into the binding of DNA to the coated surfaces and are thus an important prerequisite for the simultaneous manipulation and observation of single DNA strands. For comparison, initial pulling measurements were performed with λ-phage BSTE digest DNA deposited on gold (Figure 1). After straightening the DNA chains to the full contour length, an additional force of 65 pN resulted in the B-S overstretching transition, during which the DNA extends to 1.6 times the contour length.[6] The B-S transition is followed by a second shoulder at forces around 300 pN. This was previously attributed to dehybridization of the unattached single strand of the DNA.[8] After reproducing this well-known behavior, we examined the binding of the same λ-phage DNA to two different transparent surfaces, namely, polylysine-coated glass and silanized glass. The two surfaces differ pronouncedly in their binding properties. In pulling experiments with DNA on polylysine-coated surfaces, pulls significantly exceeding the contour length of the BSTE II digest fragments (size distribution 40–2900 nm) are commonly observed. However, these curves rarely afford the characteristic B-S transition plateau. Frequently, long pulls with uncharacteristic force curves were measured (inset to Figure 1 b). Only in very few cases could the force curves be unambiguously assigned to DNA due to their B-S plateau (Figure 1 b). To determine whether the polylysine itself is pulled off the surface instead of the λ-phage DNA, we performed cross-checking experiments on pure polylysine-coated surfaces without adding λ-phage DNA. In this case, no pulling events were detected. Our results can be explained by the formation of DNA–polylysine aggregates.[19] Due to protonation of the amino groups, polylysine is highly positively charged. Therefore, strong electrostatic interactions with the negatively charged phosphate groups of DNA are expected. The rupture forces are frequently high, which reflects the expected strong adhesion of the aggregates to the surface. However, from the force-spectroscopic data alone it is not possible to derive information about the structure of the aggregates (see section on imaging below).

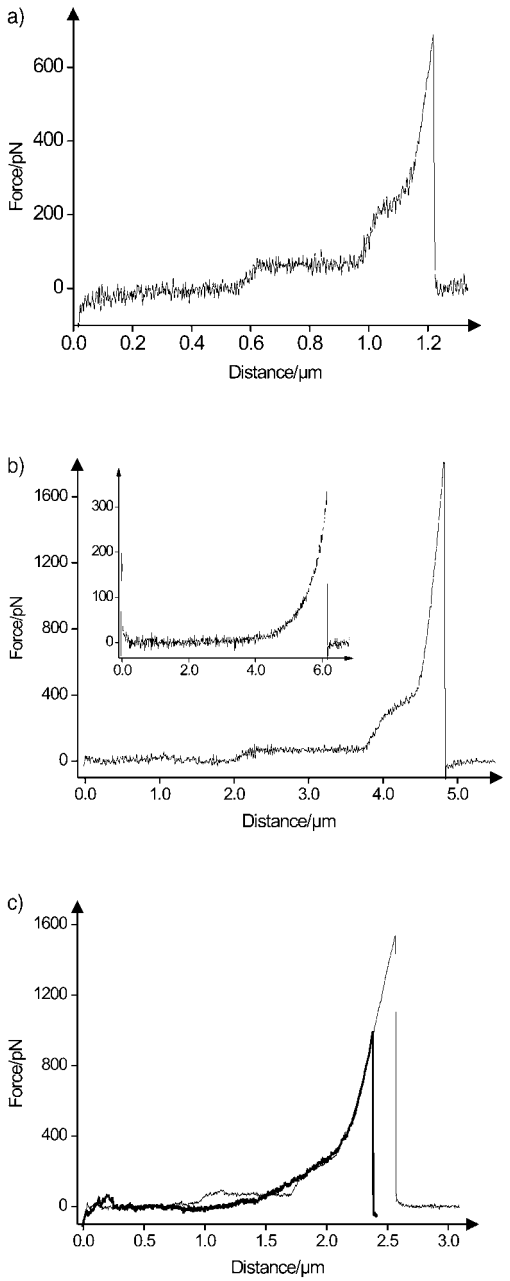

Figure 1. *Typical force–distance curves of DNA on different surfaces. a) Pulling a DNA strand from a gold-coated surface. b) Pulling a DNA strand from a polylysine-coated glass surface. The main plot shows a characteristic DNA curve with a plateau and shoulder, while the inset depicts the more frequently observed featureless force curve attributed to DNA/polylysine aggregates. c) Pulling DNA from a silanized glass surface. The typical structures observed for unlabeled DNA (black curve) disappear after labeling with TO-PRO-3 (gray curve).*

In contrast to polylysine, the silanized surfaces are highly hydrophobic and regularly exhibit characteristic force curves of λ-phage DNA when the latter is deposited at pH 5.5 and measured at pH 8.0. From the force spectroscopic experiments on silanized glass, 131 events were recorded that show the typical

B-S transition plateau at 68 ± 14 pN and can thus clearly be attributed to single DNA strands (Figure 1c). Comparatively high absolute pulling forces of up to about 1.5 nN, with an average value of 375 pN were observed. The determined pulling length distribution with a mean of 754 nm and only three events with rupture lengths above 3.5 μm are in accordance with the contour length distribution of the BSTE II digest DNA fragments. Our results support the model proposed by Allemand et al.,[18] who suggest that low pH values lead to partial melting of the DNA and exposure of the hydrophobic core, which subsequently binds to hydrophobic surfaces. We find that the associated binding strength of DNA to silanized glass is comparable to that observed on Au surfaces. Since we were interested in performing experiments on fluorophore-labeled DNA, the influence of an intercalating dye on the mechanical behavior of single DNA strands was also investigated. We chose the silanized surfaces for these experiments, because the B-S transitions were more likely to be detected on these (see above). TO-PRO-3 was used as intercalating fluorophore at a concentration of 5×10^{-6} M dye in buffer. At this label concentration, there is a clear loss in cooperativity, which has been reported previously for different intercalators.[16,20–22] The plateau at 65 pN is now replaced by a gradual increase in force with increasing pulling length (see Figure 1). No significant effect on the rupture force (mean of 394 pN), the rupture length (average of 811 nm), or the slope of the force curve after the melting transition was observed in 131 registered events.

Fluorescence Imaging

To gain a more thorough understanding of the interaction of DNA with different surfaces, we investigated the binding of TO-PRO-3-labeled λ-phage BSTE II digest DNA on both polylysine-coated and silanized glass surfaces using a wide-field fluorescence imaging setup. Generally, on polylysine no mobility of the DNA is detected after deposition. Frequently, however, the DNA aggregates to form larger condensed structures on the surface.[19] Different degrees of aggregation, ranging from DNA bundles branching into individual DNA strands, over dumbbell-like structures, to globular aggregates, are easily identified (Figure 2). In contrast to this, DNA strands deposited on silanized surfaces are only attached at specific single points along the chain. Thus, movement of the main part of the chain is still possible. Shorter DNA strands are frequently observed to undergo free rotation in solution around a fixed end pivot (Figure 3). Surprisingly, DNA strands with lengths far beyond that of the longest BSTE II fragment are also detected. In one case, a strand with an overall length of more than 300 μm was observed (Figure 4). These long strands, which are well suited to AFM manipulation experiments, are generated by a switch in pH from 5.5 to 8. The observations made in these imaging experiments confirm the results of the force spectroscopic measurements. On polylysine, the electrostatic interaction between DNA and polylysine leads to the formation of large aggregates. These bind strongly to the surface over the whole strand length. In contrast, on the silanized surfaces, the DNA attaches only at certain points. This pointwise attachment is in accordance with the model proposing partial exposure of the hydrophobic core of the DNA. Hydrophobic sections are also present at the termini of λ-phage DNA in the form of a single strand overhang of unpaired bases. Interactions at the respective ends of different

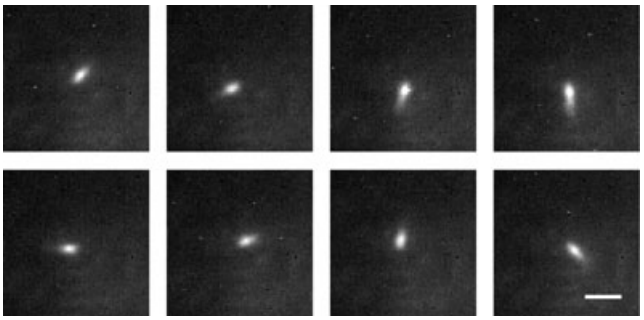

Figure 3. *Snapshots from a movie showing the free rotation of a dye-labeled DNA strand attached at one end to a silanized surface. The free tail of the DNA chain can be seen as a slightly darker arm randomly orbiting around the bright central anchor point. This confirms the single-point attachment of DNA to the silanized surface. The images are taken from a 140 s movie sequence. Scale bar: 3 μm.*

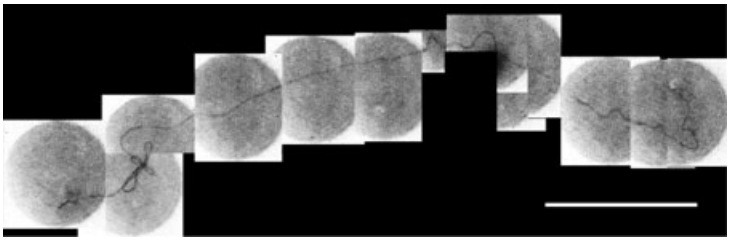

Figure 4. *Fluorescence images of a TO-PRO-3-labeled superlong DNA chain formed from BSTE II digest DNA on a silanized surface. Polymerization of the fragments is induced by a pH shift from 5.5 to 8. Scale bar: 50 μm.*

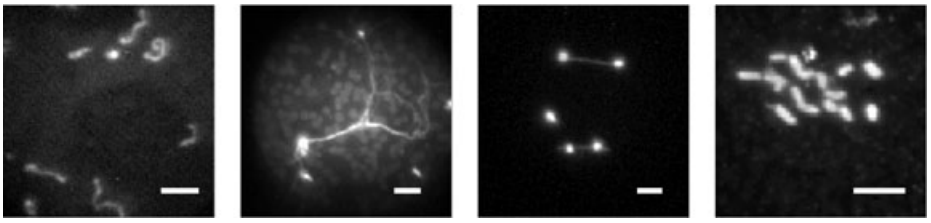

Figure 2. *Fluorescence images of TO-PRO-3 labeled λ-phage DNA deposited on polylysine coated glass surfaces. The various structures that are observed include, from left to right single DNA strands, DNA bundles, DNA dumbbells, and DNA globules. These structures correspond to different condensation products of DNA with polylysine. Scale bars: 5 μm.*

DNA fragments could therefore also be responsible for the formation of the long DNA chains, possibly by partial denaturation at pH 5.5 and subsequent rehybridization of complementary ends at pH 8.

Simultaneous Force Spectroscopy and Fluorescence Imaging

The different binding properties of the two surfaces enable two types of experiments using simultaneous AFM manipulation and fluorescence imaging. For this purpose, the MFP was mounted on top of the inverted microscope. The first type of combined AFM/fluorescence experiment makes use of the strong binding of the DNA/polylysine aggregates over the whole length on the highly attractive polylysine-coated surface. By directed deposition and fixation of the DNA, micrometer-sized structures can be generated (Figure 5). As seen in the imaging experiments, larger DNA agglomerates are frequently observed on the surface. Positioning an AFM tip on top of these agglomerates can transfer material to the tip. By bringing the AFM tip into contact with the surface, this material can subsequently be deposited. The deposition rate is constant with time, as is shown in Figure 5. In this image series the AFM tip was held at a constant distance from the surface, while the piezo scanner was employed to move the sample underneath ($v \approx 0.3\ \mu m\,s^{-1}$). Except for the turning points, where the tip was halted for a few seconds, the fluorescence intensity of the straight lines drawn is uniform. By changing the direction of the piezo movement, DNA patterns can be written on the surface until depletion of the material on the tip. The technique presented here is similar to the recently introduced dip-pen method.[23,24] The latter uses capillary forces to transfer molecules between an AFM tip covered with the deposition material and a surface. With this method it is possible to generate patterns with a variety of different small molecules. However, the dip-pen technique can not be employed in solution, which may be detrimental to the integrity of many biomolecules, such as proteins and DNA. Compared to fixation to polylysine-coated surfaces, binding of DNA to silanized glass is different in two respects. On the one hand, aggregation is very much reduced, so that attachment of single DNA strands becomes possible. On the other hand, binding to the surface is limited to specific points. The described preparation of DNA on silanized surfaces leads to isolated DNA strands. These can be manipulated in a fashion similar to the experiments described for pattern generation on polylysine surfaces. In this case, however, no DNA must be transferred to the AFM tip. Instead, the deposited DNA can be pushed directly by laterally moving the sample under the stationary AFM tip, which is in contact with the surface. Experiments of this kind once more confirm the pointwise attachment of the DNA chain. An example is shown in Figure 6. A DNA strand, which was previously fixed to the surface at two points is stretched by moving the sample under the AFM tip. In contrast to the experiments on polylysine, the DNA snaps back into its original position on slipping away from under the tip. Thus, on the silanized surface only negligible attraction to the surface occurs over the strand length. Occasionally, stretching the DNA and holding the AFM tip at a specific position, will lead to the attachment of the strand at this location. We explain this observation by tip-mediated partial dehybridization of the double helix and the resulting hydrophobic interaction with the surface. However, these events are rare and have restricted use for controlled patterning.

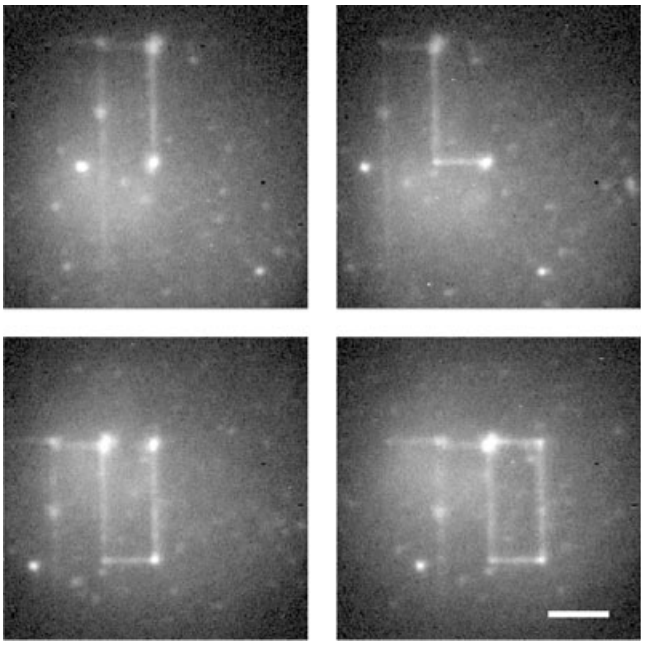

Figure 5. *Simultaneous writing and imaging of micrometer-sized structures with TO-PRO-3-labeled DNA on a polylysine-coated glass surface (sequence from upper left to lower right). The structures were written by moving the glass surface under a stationary AFM tip loaded with labeled DNA. At the turning points the sample movement is halted for several seconds. As a consequence, more DNA was deposited at these points. Scale bar: 5 μm.*

The fact that the DNA strand is only attached to the surface at specific locations can be utilized to test the binding of the intercalating dye to the DNA under tension. Therefore, we analyzed the fluorescence intensity along the strand as a function of applied horizontal force. Although the force is not measured directly in this lateral pulling experiment, it can be derived indirectly from the stretching factor of the strand. The length of the relaxed strand between the fixation points is known from the fluorescence images. The extension factor of the DNA molecule is therefore given by the increase in the total chain length, which is the sum of the lengths between the AFM tip and the respective fixation points (Figure 6). The applied external force can now be derived from the total extension factor by comparison with the standard DNA force curves measured previously. An analysis of the fluorescence images shows that no additional DNA is pulled in from above the upper attachment point, and this allows the assumption that only a well-defined part of the DNA is extended (Figure 7). We find a linear decrease in the fluorescence intensity for extension factors of 1.25 to 1.55 times the contour length, equivalent to a maximum applied force of about 70 pN (Figure 7). This would approximately correspond to the B-S transition force in unlabeled DNA. A significantly different binding behavior of the in-

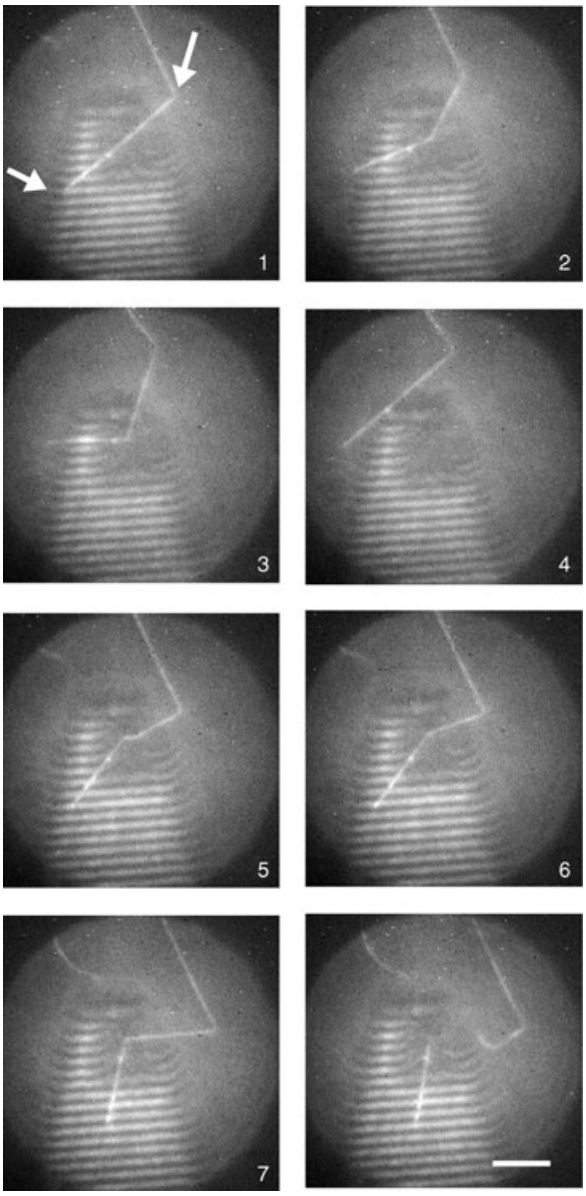

Figure 6. Simultaneous AFM manipulation and fluorescence imaging of a single TO-PRO-3-labeled DNA strand on a silanized glass surface (selected images from a longer series). The DNA strand was previously stretched and attached to two anchor points as indicated by the arrows (the interference pattern originates from the cantilever shank). By moving the sample under the stationary tip, the DNA stand can be stretched repeatedly in an arbitrary direction. In the first three images the strand is stretched downwards. At a certain extension (image 4), it slips away under the tip and springs back into its original position. Images 5 through 7 describe the subsequent stretching cycle in the reverse direction. Finally, in the last image (image 8) the DNA strand ruptures at the tip. Scale bar: 5 μm.

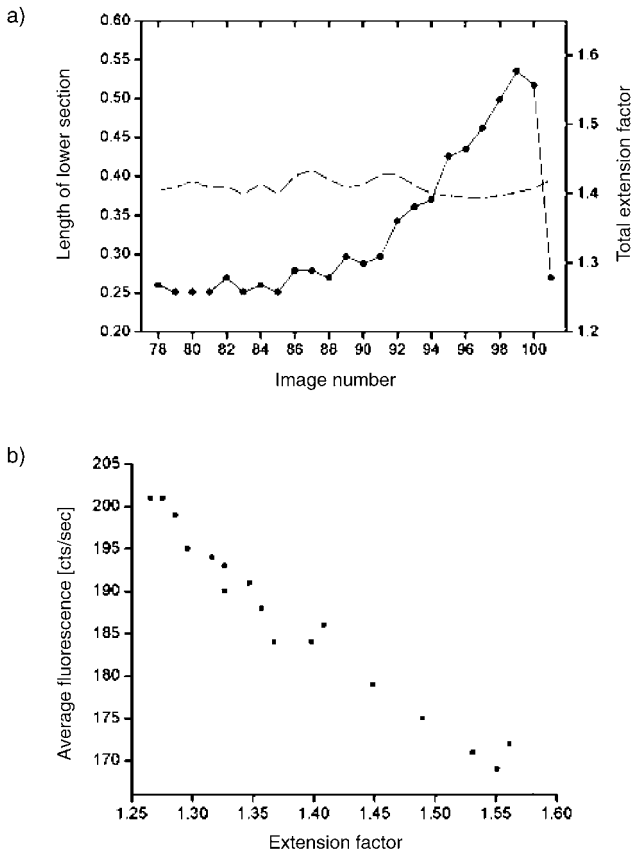

Figure 7. Dependence of the fluorescence intensity of a TO-PRO-3-labeled DNA strand on strand extension during the lateral extension experiment described in Figure 6. a) The graph with the filled circles describes the total extension of only that part of the DNA strand which is fixed between the points indicated by the two arrows in Figure 6. The empty squares depict the length ratio of the DNA sections defined by the tip and each of the two fixation points marked in Figure 6. This ratio is constant during stretching and independent of the total extension factor, that is, no additional DNA is pulled into the relevant section between the arrows. b) Dependence of the fluorescence intensity of TO-PRO-3-labeled DNA on its extension. The average intensity was determined for the section between the tip and the upper arrow in Figure 6.

tercalator to the DNA was expected after stretching the DNA through the B-S transition. The observed linear decrease is, however, most easily explained by a mere dilution effect of the dye in the stretched probe length. Unfortunately, we have so far not succeeded in extending the DNA beyond the B-S transition plateau. The reason for this is that at higher pulling forces either the strand slips away from under the bent AFM tip or the attachment at one of the fixation points breaks. On several occasions we also observed rupture of the strand by the AFM tip, as is depicted in Figure 6. These rupture events are the same as those observed in the force spectra obtained with atomic force spectroscopy. To our knowledge, this is the first time that the scission of a single polymer strand (in our case the DNA molecule) has directly been observed in an optical experiment. The simultaneous optical visualization enables a detailed description of the rupture process. While in most cases the DNA breaks at the AFM tip inducing the lateral displacement, we rarely detected rupture events at other positions along the strand (data not shown). All rupture events observed in our experiments occur at relatively low applied forces, as compared to the maximum rupture forces measured in horizontal force-spectroscopic experiments. However, force spectroscopy is biased towards events reaching the high-force regime and generally dismisses pulling curves with low forces. Nevertheless, we assume that in our case, the junctions generated along the linearly polymerized long DNA strands, which we used in these experiments, function as predetermined

breaking points and thus explain the relatively low rupture forces. Combined optical and AFM experiments which investigate the breaking mechanisms of DNA in detail are currently underway.

Conclusions

In conclusion, we have reported the simultaneous AFM manipulation and fluorescence imaging of single DNA molecules on transparent surfaces. We utilized force-spectroscopic measurements and imaging experiments to characterize the binding of fluorescently labeled DNA on polylysine-coated and silanized glass surfaces. Two distinct binding behaviors are observed: on the polylysine-coated surfaces deposition of DNA leads to the formation of aggregates which bind strongly over the whole strand length. We show that it is possible to exploit this type of attachment for writing micrometer-sized structures of DNA with an AFM tip. In contrast, only a pointwise attachment of DNA is observed on silanized surfaces. Considerably less aggregation is found under these conditions. The formation of well-defined fixation points to the silanized glass along the DNA can be used to horizontally stretch a single DNA strand with an AFM tip. The advantage of this method over vertical stretching is that the strand remains in the focal plane of the microscope at all times and can thus be imaged accurately. The results of these lateral manipulation experiments demonstrate that a DNA chain can be extended to at least 1.6 times the original contour length before the strands snap. No significant shift in the number of intercalated chromophores is observed upon stretching the DNA up to a force of about 70 pN, corresponding to the end of the B-S transition in unlabeled DNA. In many cases, a further extension of the DNA leads to strand rupture. This is the first time that such events, which are commonly encountered in force spectroscopy of polymers, have been visualized directly by real-time optical microscopy.

Experimental Section

For the preparation of λ-phage digest, a solution of 20 $\mu g\,mL^{-1}$ λ-phage digest BSTE II (117–8454 bp, Sigma, Germany) was prepared with TE buffer (Tris/EDTA composed of 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8, Fluka, Germany). The samples were obtained by overnight adsorption of a 50 μL drop of this stock solution onto the clean glass or Au surface. Unattached DNA fragments and precipitated salt were purged from the surface by washing with TE buffer. Cleaned glass coverslips were coated with polylysine (poly-L-lysine hydrobromide, 30–70 kDa; Fluka, Germany) by compressing a 5 μL drop of a 5 $\mu g\,mL^{-1}$ aqueous polylysine solution between two coverslips and then separating the surfaces after overnight drying in an evacuated desiccator. Assuming homogeneous adsorption, a polylysine density of about 100 strands/μm^2 is calculated. Coverslips were silanized by 1 d of gas-phase adsorption in a 7-octenyltrichlorosilane (Sigma, Germany) atmosphere. For measurements on silanized surfaces, the original λ-phage digest DNA was diluted with MES buffer (2-(*N*-morpholino)ethanesulfonic acid sodium salt, Sigma, Germany, neutralized to pH 5.5 with aqueous NaOH) to afford a 1 $\mu g\,mL^{-1}$ DNA stock solution. Following overnight incubation of the freshly silanized coverslips with this DNA solution and rinsing with MES buffer, the samples were then measured in TE buffer. Long DNA strands were obtained by diluting the original λ-phage BSTE digest DNA in MES buffer by a factor of 1000 to yield a 1 $\mu g\,mL^{-1}$ DNA solution. This solution was added to a freshly silanized surface and left to incubate overnight before washing and imaging in the TE buffer.

Force curves were measured in the respective buffer solution using a Molecular Force Probe (MFP, Asylum Research, CA) equipped with Si_3N_4 microlever cantilevers (ATOS, Germany). The cantilever spring constants were determined by the thermal-noise method.[25] Typically, a pulling velocity of 2 $\mu m\,s^{-1}$ was used. Wide-field fluorescence images of the labeled DNA with illumination spot diameters of 24 μm were recorded using an inverted microscope (TE300, Nikon, Japan) and an oil-immersion objective (100×, N.A. = 1.45, Zeiss, Germany). The sample coverslip was illuminated with the 633 nm line of an HeNe laser (Laser2000, Germany), and the fluorescence collected in the epi configuration. Residual excitation light was rejected with a bandpass filter (AHF, Germany) before detection of the fluorescence signal with a back-illuminated CCD camera (LaVision, Germany). λ-Phage BSTE II DNA digest samples were prepared as described above. The same buffer was used to prepare samples from a $3\times10^{-11}\,mol\,L^{-1}$ λ-phage DNA (48502 bp, Sigma, Germany) stock solution. DNA was labeled with TO-PRO-3 (Molecular Probes, OR) as an intercalating fluorophore. Stock solutions of the dye were prepared by diluting 1 mM DMSO solutions in a 7/3 glycerol/water mixture to 1×10^{-4} M before further dilution in TE buffer to a 5×10^{-6} M dye concentration. To minimize photobleaching, the coverslips were treated with TO-PRO-3 solution containing 1% Slowfade (Molecular Probes, OR). Images with 1 s exposure time and an illumination intensity of 1.2 $W\,cm^{-2}$ could be taken over a period of hours without significant loss of fluorescence. In some cases, DNA was aligned on dried polylysine-coated coverslips by spin coating prior to labeling.[26]

In the combined experiments, in order to suppress the background signal of the laser diode monitoring the deflection of the AFM tip, its spectrum was narrowed by means of a bandpass filter (AHF, Germany). A piezo-stage (Physik Instrumente, Germany) was used for lateral displacement of the sample, while a separate positioning system aligned the cantilever tip with the optical focus of the microscope. All cantilevers examined emitted a spectrally broad luminescence background, red-shifted with respect to the excitation wavelength. The cantilever background signal can be reduced by either using long AFM tips or illumination by total internal reflection.

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Keywords: atomic force microscopy · DNA · fluorescence spectroscopy · single-molecule studies

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