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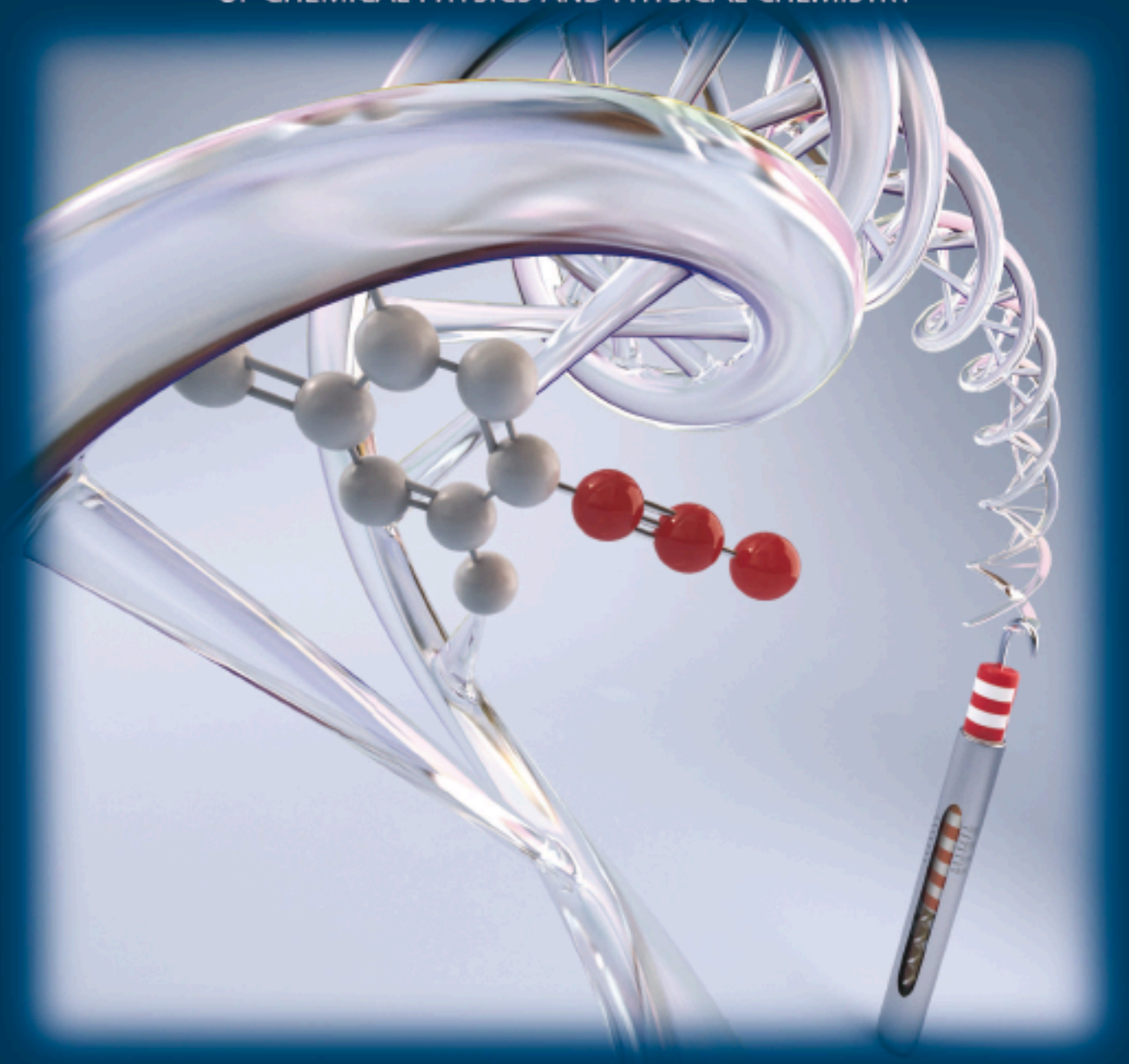

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The increase of mechanical stability of DNA that is due to the integration of propynyl bases is determined by using two complementary force spectroscopy techniques and found to be strongly dependent on incubation conditions, as described by D. A. Pippig et al. on p. 2085. Cover Image by Christoph Hohmann, Nanosystems Initiative Munich (NIM).

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CHEMPHYSCHEM Communications

C-5 Propynyl Modifications Enhance the Mechanical Stability of DNA

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Increased thermal or mechanical stability of DNA duplexes is desired for many applications in nanotechnology or -medicine where DNA is used as a programmable building block. Modifications of pyrimidine bases are known to enhance thermal stability and have the advantage of standard base-pairing and easy integration during chemical DNA synthesis. Through single-molecule force spectroscopy experiments with atomic force microscopy and the molecular force assay we investigated the effect of pyrimidines harboring C-5 propynyl modifications on the mechanical stability of double-stranded DNA. Utilizing these complementary techniques, we show that propynyl bases significantly increase the mechanical stability if the DNA is annealed at high temperature. In contrast, modified DNA complexes formed at room temperature and short incubation times display the same stability as non-modified DNA duplexes.

In recent years, DNA has emerged as a prominent nanoscale building block. It exhibits unparalleled properties such as the ability to self-assemble depending on its sequence, which can be designed as required. Thus, two- and three-dimensional defined structures such as scaffolded DNA origami[1] can be created at the nanoscale. Another example are small "DNA bricks",[2] which can be assembled to larger structures in a LEGO-like fashion. However, materials that are prepared using DNA harbor the drawback of only limited thermal stability. In general, DNA structures cannot be employed at elevated temperatures in solution as they disassemble at high temperatures. In order to overcome this disadvantage, the heat tolerance of DNA structures can, for example, be improved by about 30 °C by photo-cross-linking.[3] For other applications, the limiting factor is the mechanical stability of DNA structures. It is not directly correlated to the structures' thermal stability, as it largely depends on the orientation in which an external force is applied. A standard example is given by a short DNA duplex. Here, a higher rupture force is observed if the duplex is melted by applying a force load in shear geometry at opposing 5′ termini than if the DNA is opened like a zipper from 5′ and 3′ termini of the same end.[4] In the latter case, one base pair at a time is loaded under force while in the first case all base pairs are stretched simultaneously. For the shearing of short DNA duplexes, the average rupture force is thus dependent on the number of base pairs (bps).[5] At rupture forces of about 65 pN a force plateau is reached. This so-called BS-transition can be attributed to an overstretching of the DNA and is already observed for DNA duplexes as short as 30 bp.[6] Internal modifications of bases are capable of altering both thermal and mechanical stability of a DNA duplex. A prominent example is the methylation of the 5′ position of cytosine.[7] Depending on the amount and position of modified bases in a DNA duplex the melting temperature[8] and the probability of strand dissociation under force are altered, as methylation can both stabilize and destabilize DNA duplexes.[9] Another alternative is, for example, the use of salicylic aldehyde nucleosides, which confers strong mechanical stabilization upon copper complexation.[10]

In order to reach higher mechanical stability, integration of bases modified with a propynyl group at the 5′ position of pyrimidines[11] is promising, as the apolar planar group extends into the major groove and enhances base-stacking. Graham et al.[12] determined the thermodynamic parameters for a 12 bp DNA duplex containing five propynyl bases compared to an unmodified duplex with UV-melting studies: the significant decrease in enthalpy is attributed to the electronic interactions in base-stacking and counteracts the entropy decrease likely resulting from more ordered water molecules normally found in the major groove. This results in a decrease in free energy ΔG and thus a stabilized complex.[12] Compared to other base modifications such as methylation, the incorporated propynyl bases lead to an even higher increase in melting temperature per base,[13] number and position of the propynyl bases playing an important role.[14] Higher mechanical stabilities would be useful to render DNA nanostructures more stable in the presence of external forces, for example, for techniques such as the molecular force assay (MFA), where the mechanical stability of a molecular complex is determined by comparing it to a known DNA reference complex. An increase in mechanical stability of the DNA duplex broadens the dynamic range of the assay and enables, for example, the characterization of protein–protein interactions.[15]

Herein, the MFA technique is employed together with atomic force microscope (AFM) based force spectroscopy to

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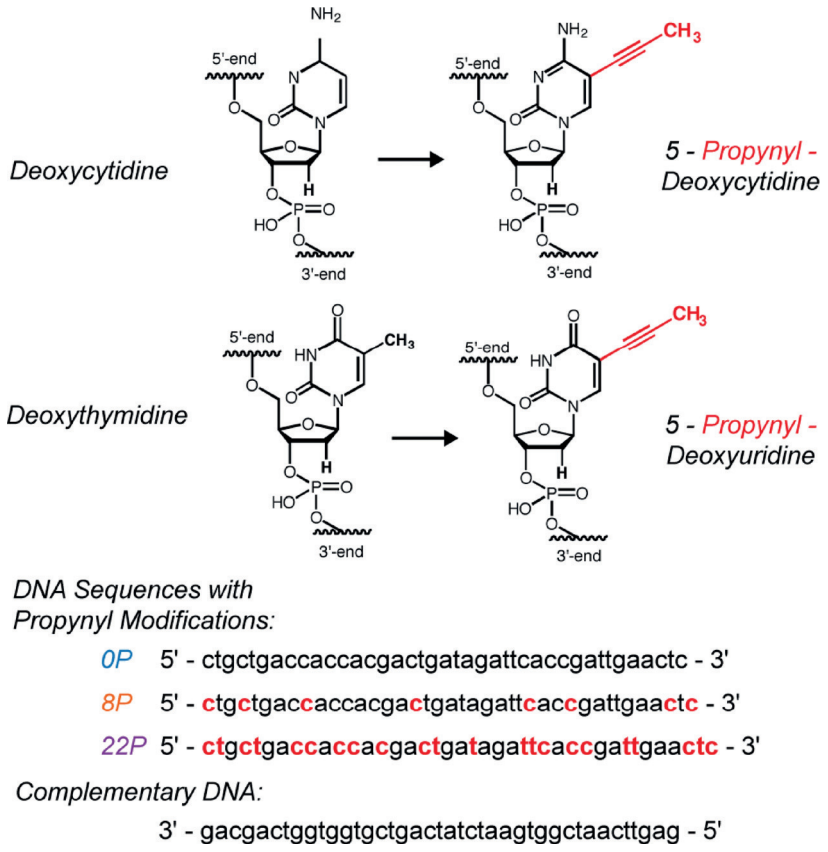
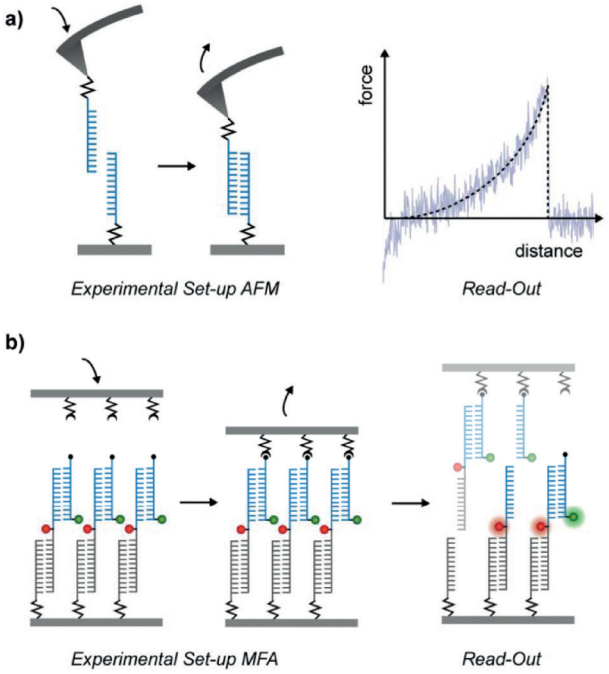


Scheme 1. Propynyl bases and DNA sequences. In order to obtain propynyl bases, the 5′ position of the pyrimidines cytidine or thymidine is modified with an additional propynyl group, which extends into the major groove of the DNA helix. A stabilization of the DNA duplex harboring propynyl bases is thus expected to result from enhanced base-stacking. DNA oligonucleotides with the same sequence but a different number of propynyl bases, namely none (*0P*, blue), 8 (*8P*, orange) and 22 (*22P*, purple) are investigated.

characterize the difference in mechanical stability of short DNA duplexes with varying numbers of integrated propynyl bases. Three different 40-base-pair-long oligonucleotides are investigated in shear mode, harboring no modification (0P), eight propynyl bases (8P) and 22 propynyl bases (22P), respectively (Scheme 1). The sequence is identical for all three strands, enabling binding to the same complementary DNA strand. A stabilization of the DNA complex to average rupture forces higher than the 65 pN that can be reached with unmodified DNA is desired. Therefore, the length of 40 bps is chosen for the duplexes. Two complementary force spectroscopy techniques are employed to characterize the DNA duplexes. The basic principle of the measurement with the atomic force microscope (AFM)[6,16] is displayed in Scheme 2 a. The two strands are attached covalently via PEG spacers to the lower surface and the cantilever, respectively. Upon lowering the cantilever onto the glass slide, the DNA duplex to be investigated is formed. Retraction of the force-calibrated cantilever stretches the PEG linker and the DNA duplex until it finally ruptures, as depicted in the resulting example force–distance curve (Scheme 2 a). The force resolution is limited due to thermal fluctuations by the size of the cantilever, which acts as the force sensor. In MFA[17] the size of the force sensor is minimized to a second DNA duplex. As shown in Scheme 2 b, this reference duplex is coupled in series with the duplex to be investigated and clamped between two surfaces. Retraction of the upper surface compares the mechanical stability of both complexes directly until, statistically, the weaker one ruptures. The outcome of the experiment is given by the position of the fluorophore dye on the linker after force load, as it stays with the stronger duplex. A second dye on the uppermost DNA strand forming a FRET pair with the dye on the linker allows for correction of constructs that did not couple to the upper surface and have thus not been under force load. The main advantage of the MFA technique lies in the parallelization of force-spectroscopy experiments. About 10^4 complexes per μm^2 are tested simultaneously.[18] An important difference between the two techniques is the incubation time and condition of the duplex to be investigated. While for the AFM experiment the incubation time of the duplex depends on the contact time of the cantilever with the surface, the duplex in the MFA experiment is pre-incubated overnight and can also be annealed with a temperature ramp starting from denaturing temperatures.

In order to determine if integration of propynyl bases leads to average rupture forces higher than for unmodified DNA, AFM experiments were performed. To exclude calibration uncertainties, all measurements were conducted with the same cantilever harboring the complementary strand, while the strands 0P, 8P and 22P were covalently attached to the surface in three distinct populations. Representative histograms for data obtained with a retraction velocity of 1000 nm s^{-1} are displayed in Figure 1. The histograms are fitted with the Bell–Evans model (Supporting Information) and the most probable rupture forces were 65.1 ± 4.5 pN (0P; $N = 705$ curves), 65.5 ± 4.4 pN (8P; $N = 579$) and 64.7 ± 4.5 pN (22P; $N = 1079$), respectively. Thus, the most probable rupture forces of 0P, 8P and 22P cannot be distinguished within the error bars. The same conclusion holds true for the other tested retraction velocities of the cantilever (the corresponding data can be found in the Supporting Information). However, although the most probable rupture forces were indistinguishable within error, we performed pair-wise two-sample Kolmogorov–Smirnov tests, in order to test the hypothesis whether the rupture force distributions are significantly different. For all retraction velocities



Scheme 2. Experimental setups of AFM and MFA. The DNA duplexes are investigated with two complementary single-molecule force spectroscopy techniques. To this end, all three DNA strands are hybridized to the same, unmodified complementary strand and integrated into the experimental setups of the AFM (a) as well as the MFA (b). In the well-established AFM force spectroscopy, the two DNA strands of the duplex are covalently attached to a lower glass surface and a cantilever, respectively. The duplex to be investigated (blue) forms when the cantilever is lowered onto the glass surface. Retraction of the force-calibrated cantilever yields a force-distance curve as the outcome of the experiment. The cantilever of the AFM experiment can be regarded as an elastic spring and acts as the force sensor. In contrast, in an MFA experiment, the force sensor is given by a second DNA duplex (grey), which is coupled in series with the duplex to be investigated (blue). Those DNA constructs are built up on a glass slide and then clamped between two surfaces via a biotin–streptavidin interaction (b). Retraction of the upper surface builds up a force acting on both molecular complexes until, statistically, the weaker one ruptures. The outcome of the experiment is read out via a fluorophore (red circle) attached to the linker between the two duplexes, which only gives a signal if the lower reference complex is still intact after rupture. A second fluorophore coupled to the upper strand (green circle) is necessary for the correction of the analysis if the molecular complexes did not couple to both surfaces and thus have not been under force load.

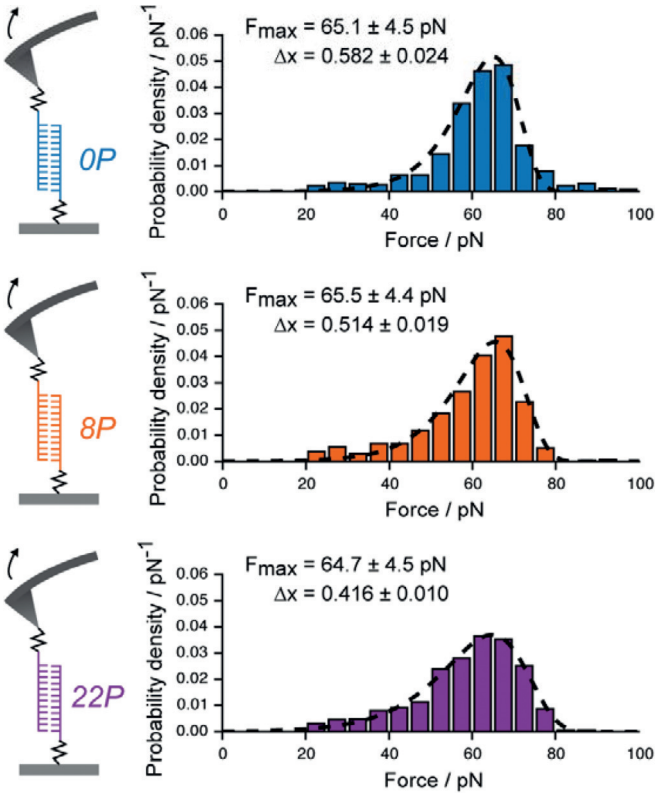


Figure 1. Investigation of DNA duplexes containing propynyl bases with the atomic force microscope. Representative histograms of the most probable rupture force for a retraction velocity of the cantilever of 1000 nm s^{-1} are shown for all three DNA complexes with a varying amount of propynyl bases. The most probable rupture forces F_{max} are all within error in the vicinity of the BS-transition (65 pN). They were determined by fitting the histograms within the Bell–Evans formalism.

besides 500 nm s^{-1}, the rupture force distributions for 8P and 22P were significantly different from the 0P distribution with a p value below 0.05. Hereby, the p values of the 22P distributions are considerably smaller than that those of the 8P distributions, when compared against the 0P distributions. This can also be seen in the width of the rupture force distribution, which increases with the number of propynyl modifications.

The Bell–Evans fits to the rupture force distributions confirm the validity of the model for this data and allow for the determination of the distance to the transition state in the binding energy landscape. We found for the three modified duplexes 0P, 8P and 22P a Δx of 0.582 ± 0.024 nm, 0.514 ± 0.019 nm, and 0.416 ± 0.010 nm respectively.

Figure 2 displays the characterization of the same sequences with MFA. In order to make the data directly comparable, all duplexes in question are tested against identical reference DNA. The normalized fluorescence (NF) gives the ratio of still intact reference duplexes after force load in comparison to the initial amount of assembled molecular constructs after correction for background and complexes that have not been under force load. Thus, a decreased value of the NF results from a strengthened duplex in question. With the MFA, the duplexes with 0P, 8P and 22P oligonucleotides were tested in two variants: for one sample the duplexes were pre-incubated at room temperature (RT) overnight, for the other they were annealed by heating to 95 °C and cooling to 5 °C over four hours. We determined the following results for the NF mean values and error bars: $NF_{RT}(0P) = (0.341 \pm 0.007)$, $NF_{RT}(8P) = (0.327 \pm 0.014)$, and $NF_{RT}(22P) = (0.316 \pm 0.013)$ for the incubation at RT as well as $NF_{95}(0P) = (0.344 \pm 0.011)$, $NF_{95}(8P) = (0.306 \pm 0.012)$, $NF_{95}(22P) = (0.262 \pm 0.017)$ for the annealed complexes. The respective results for the two samples are depicted in Figure 2. For the duplexes incubated at RT (right bars), a slight stabilization depending on the number of modifications is discernible, although within standard deviation error bars. In contrast, for the duplexes annealed at high temperature (left bars), the stabilization effect is significant.

The MFA determines the relative stability of the DNA duplex in question by comparing it to a DNA reference duplex during

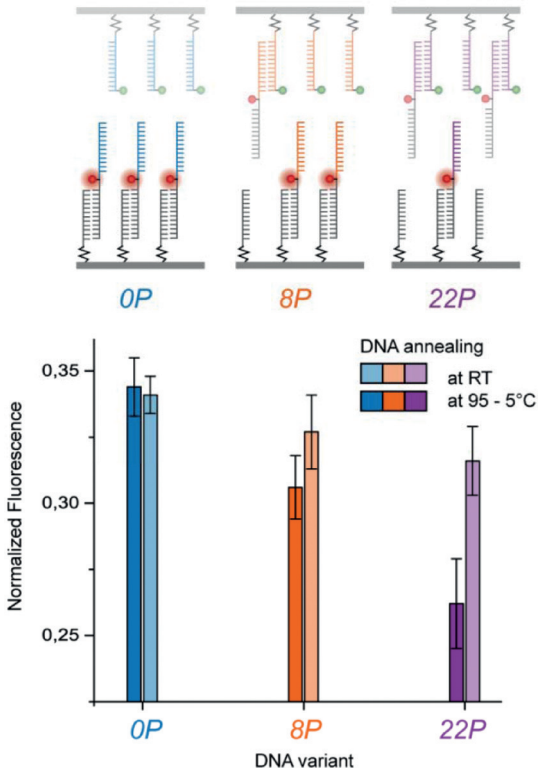


Figure 2. Investigation of DNA duplexes containing propynyl bases with the molecular force assay. In contrast to the AFM experiment, the DNA duplexes are not formed when the two surfaces are brought into contact, but instead the whole molecular construct consisting of both duplexes in series is build up in advance onto the lower glass slide. Hereby, the upper complex can be pre-incubated before attaching it to the surface. The more stable an upper complex is when compared to the same reference duplex, the less fluorescence signal remains on the lower glass slide after force load, as the fluorophore remains with the stronger duplex. This means that the NF value of the surface becomes smaller the higher the mechanical stability of the upper complex is. The NF values of all three DNA duplexes are displayed, with the upper complex pre-incubated by either heating up to 95 °C and cooling it down very slowly (4 h) to 5 °C (left bars) or overnight at room temperature (right bars) (all given with standard deviation error bars). Whereas the mode of pre-incubation does not influence the stability of the unmodified DNA strand *0P*, for *8P* and *22P* the stabilization trend depending on the number of propynyl bases is the same but statistically significant only for the slowly annealed DNA.

strand separation. In comparison to the duplex with the unmodified DNA, 0P, the probability of strand separation in the annealed 8P sample is altered by about [NF(8P)−NF(0P)]/NF(0P) = −11 % and by about −24 % for the annealed 22P duplex. The parallel measurement of the three samples with the MFA ensures identical measurement conditions and renders the obtained differences in rupture probability highly reliable. In the AFM measurements as well, care was taken to minimize measurement variations. In the characterization of the mechanical stability of methylated DNA conducted by Severin et al.[9] with both AFM and MFA, the experiments led to the same results for stabilizing and destabilizing effects. We thus attribute the differing results of the AFM from the MFA measurements in this case of propynyl-modified DNA to different conformations of the DNA, resulting from the different incubation conditions, particularly the temperature and time span. In the AFM experiments, the duplex forms at RT during the contact time of the cantilever to the surface, which is below 0.1 s. Longer contact times to enable longer incubation times for the duplex are not feasible, as this tremendously reduces the probability to obtain single DNA binding events. The AFM measurements yield distinct populations of rupture force for all three samples, and sequence compatibility allows for one binding mode only. The slight broadening of the force distribution width with increasing number of base modifications leads to an elevated number of events both with lower and higher rupture force. The higher variance of the modified DNA distribution might be attributed to short-lived perturbations in duplex formation caused by the propynyl modifications. However, this effect is very small. This leads to the assumption that even though the DNA duplex forms during the measurement, the short contact time is not sufficient to acquire a conformation in which the propynyl group can stabilize the DNA significantly. In support of this assumption, the results for the MFA measurement with samples incubated at RT also only show a very slight, not significant, stabilization effect. This indicates a complex energy landscape and a high potential barrier that needs to be overcome in order to form the stabilized complex. The fact that the stabilized complex is formed upon annealing at high temperature might be due to an increase in kinetic degrees of freedom under these conditions. Double-stranded DNA harboring more G–C base pairs is thermally more stable due to base-stacking interactions[19] and it unbinds at a higher external force along the long axis of the DNA.[4] It is thus a valid assumption that enhanced mechanical stability of annealed propynyl DNA is due to its increase in base-stacking interactions.

In summary, we have demonstrated that the modification of pyrimidines with a propynyl group at their 5′ position can have a significant stabilization effect on DNA duplex strand separation and thus on its mechanical stability. However, to obtain the conformation of higher stability, the DNA has to be pre-annealed at high temperature. Provided that heating of the sample is possible, propynyl-modified pyrimidines can be employed to enhance the mechanical as well as thermal stability of double-stranded DNA. For DNA origami structures that in general are also prepared by annealing, it has been shown that folding to the desired structure occurs at a narrow temperature range and can consequently also be achieved at constant temperatures specific for the structure.[20] In this context it might be possible to adjust the annealing process for propynyl-modified DNA for temperature-sensitive samples. The propynyl modification offers the advantage of standard sequence recognition, easy availability and the lack of additional treatments, such as irradiation with light. Notably, the characterization of the propynyl-modified DNA with the AFM alone would not have given the whole picture, as it is not possible to measure a statistically sufficient dataset with pre-annealed DNA. The additional measurement with the MFA technique provided crucial complementary information on the properties of the modified DNA.

Experimental Section

Atomic Force Microscopy

AFM-based force spectroscopy has been applied for analyzing the unbinding forces of the described DNA oligonucleotides comparable to Ref. [6]. The DNA strands with different propynyl modification levels were covalently coupled via PEG spacers to the probed sample surface, whereas the complementary DNA oligonucleotides were attached in the same manner to a BL-AC40TS-C2 cantilever (Olympus, Tokyo, Japan). For probing the DNA, the functionalized cantilever is brought into contact with the surface and withdrawn at different retraction velocities, ranging from 200 to 10000 nm s^{-1}. A low molecular surface density prevents the formation of multiple bonds between surface and cantilever tip. All measurements of the shown dataset were conducted with the same cantilever on one surface to ensure high comparability for different retraction velocities and DNA modification levels. In order to obtain single DNA binding events, the experiments feature no additional contact time of the cantilever on the surface before retraction. Force curves representing multiple bonding, nonspecific adhesion of molecules to the cantilever tip, or lack of interaction, were filtered out using an automated pattern-recognition algorithm. Only single worm-like chain force responses with a persistence length in the range of 0.1–0.5 nm and a contour length matching that of the DNA strands were extracted for further analysis. Rupture forces for each distinct retraction speed were plotted in histograms and fitted with the Bell–Evans model[21] to determine the most probable rupture force analogous to the analysis described in Ref. [6]. To obtain measurements over a broad range of different loading rates, several experiments were carried out for five different retraction velocities. Additionally, the standard Bell–Evans model was applied to the force versus loading rate dependency yielding the natural dissociation rate at zero force and the potential width Δx of the investigated DNA duplex (the corresponding force-loading rate plots can be found in the Supporting Information). Sample preparation and more detailed information on the measurement of rupture forces of DNA duplexes can be found, for example, in Ref. [6] and in the Supporting Information.

Molecular Force Assay

The MFA experiments have been performed as described previously, for example in Ref. [17b]. For the measurements with the MFA, three oligonucleotide strands are assembled as displayed in Scheme 2b to form two DNA helices, a reference duplex and a duplex to be investigated. The lowermost strand is attached covalently to the lower surface, a glass slide, and binds to the lower part of a longer strand harboring a Cy5 fluorophore dye at the linker between the two duplexes. The uppermost DNA strand, forming the second duplex with the longer middle strand, carries both a biotin and a Cy3 dye, forming a FRET pair with the Cy5. The upper surface consists of a soft PDMS stamp coated with streptavidin. After initial measurement of the fluorophore intensities, the stamp is lowered onto the glass slide. The biotin allows for the coupling of the uppermost strand to the stamp, so that the two DNA duplexes are clamped between both surfaces. Upon retraction of the stamp, a force builds up in the complexes and the mechanical stabilities of the duplexes are compared until, statistically, the weaker one ruptures. A second measurement of the remaining fluorescence intensities on the glass slide allows for the quantitative analysis of the experiment. The Cy5 dye attached to the linker stays with the stronger duplex. Thus, the higher the ratio of remaining intensity on the surface is to the initial intensity, the stronger the lower complex is in comparison to the upper duplex. If a molecular complex does not couple to the stamp, the DNA duplexes are not under force load and the Cy5 dye remains on the glass slide, yielding a false positive signal. This can be corrected by subtraction of the ratio of the FRET signal, which only remains if the complexes have not been under force load and the uppermost strand is still on the glass slide. The outcome of the experiment is thus given by the normalized fluorescence (NF), which denotes the ratio of still-intact lower complexes in comparison to the initial amount of complexes that have been under load. In the current standard setup, sixteen different combinations of reference and sample complex can be tested in parallel, each of them statistically significant as about 10^4 molecular complexes per μm^2 are tested simultaneously. The derivation of the equation for the normalized fluorescence and more details of the preparation, measurement and analysis process can be found in the Supporting Information. In the measurements conducted here, the oligonucleotides including the modifications were integrated as the uppermost strand, so that the upper duplex is the complex in question. The lower complex consists of a 40 bp long DNA duplex. It has a different sequence to prevent for cross-hybridization of the three strands. The sequences are given in the Supporting Information.

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Supporting Information

C-5 Propynyl Modifications Enhance the Mechanical Stability of DNA

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Supporting Information

1. Supplementary Materials and Methods

DNA Oligonucleotides

Propynyl bases can be obtained from pyrimidines, which are modified with an additional propynyl group at the 5' position of the base (see scheme 1). In desoxycytidines, this is achieved by replacing the H- group with the propynyl group. Desoxythymidines are replaced by desoxyuridines modified with the propynyl group, as uracil does not already harbor a methyl group at its 5' position as thymidine.
Experiments were performed with three degrees of propynyl bases: one strand contained no base modification (0P), one eight propynyl-desoxycytidines (8P) and the last 13 propynyl-desoxycytidines as well as nine propynyl-desoxyuridines yielding 22 propynyl bases (22P). The modifications are distributed over the same sequence of 40 bases. The unchanged base-recognition for propynyl-modified bases yields binding of all examined oligonucleotides to the same complementary strand. All measurements in this study are performed at room temperature and physiological salt concentrations in 1xPBS buffer.

MFA Preparation

The lower surface with the two DNA duplexes in series was prepared as described previously *e.g.* [1] except for small modifications. The DNA oligomers were purchased including all modifications from biomers.net GmbH (Ulm, Germany) and IBA GmbH (Göttingen, Germany).
The lowermost oligonucleotide strand was coupled covalently *via* its NH_2-group at the 5' end to the aldehyde-functionalized glass slide (Schott GmbH, Jena, Germany). Five hexaethyleneglycol (HEGL) linkers acted as additional spacers. In the middle strand, a Cy5 fluorophore is attached to the poly-t-linker connecting the sequences for the two complexes. The direction of the middle strand is inverted in the linker, ensuring that both complexes are probed from the 5' ends. The three different uppermost strands harbor varying amounts of propynyl modification. Additionally, each strand carries a Cy3 fluorophore forming the FRET pair with the Cy5 dye in the middle strand as well as a biotin on the 5' end for coupling to the upper surface.

Lower Strand
NH2 - 5xHEGL - 5'- $(t)_{10}$ – ctg atg agt cga caa cgt atg cac tac gct cgc tta cta g
Middle Strand
3' - gac gac tgg tgg tgc tga cta tct aag tgg cta act tga g - $(t)_7$ - 5' - **(Cy5)** - 5' - $(t)_7$ - cta gta agc gag cgt agt gca tac gtt gtc gac tca tca g -3'
Upper Strands
(0P) Biotin - 5' - $(t)_{10}$ - ctg ctg acc acc acg act gat aga ttc acc gat tga act c - 3' - **(Cy3)**
(8P) Biotin - 5' - $(t)_{10}$ - **c**tg **c**tg ac**c** acc acg a**c**t gat aga tt**c** ac**c** gat tga a**c**t **c** - 3' - **(Cy3)**
(22P) Biotin - 5' - $(t)_{10}$ - **c**tg **c**tg a**cc** a**cc** a**c**g a**ct** ga**t** aga **ttc** a**cc** ga**t** **t**ga a**ct** **c** - 3' - **(Cy3)**

The lower strand was spotted in 1 µl droplets of 25 µM in 5xPBS (Roche Life Science, Indiana, USA) in a 4x4 pattern on the functionalized glass slide and incubated in a saturated NaCl ddH_2O atmosphere overnight. The resulting Schiff Bases were reduced with 1% aqueous $NaBH_4$ (VWR Scientific GmbH, Darmstadt, Germany) for 90 minutes to render the attachment covalent. After a washing step with ddH_2O the slide was incubated in 1xPBS with 4% BSA (bovine serum albumin; Sigma-Aldrich GmbH, Munich, Germany) to reduce unspecific binding. A custom-made silicone isolator with 16 wells (Grace-Biolabs, OR, USA) was positioned on top of the spotted pattern of the lower DNA strand. A pre-incubated mix of middle and respective upper strand was spotted in the wells and incubated for 1h. The ratio of middle to upper strand was 1:2 (100nM:200nM) in 5xPBS to ensure a saturation of middle strands with bound upper strands. The mix was either incubated over night at room temperature (RT) or annealed by heating to 95°C and cooling slowly over 4 hours to 5°C. In order to remove free unbound DNA, the slide was rinsed carefully in washing steps with 2x, 0.2x and 1xPBS after removal of the isolator.

The upper surface, a soft PDMS (polydimethylsiloxane) stamp with 16 pillars matching the pattern of DNA constructs on the glass slide, is custom-made and functionalized in our lab as described in detail *e.g.* in [1]. The pillars are 1 mm in height and 1.1 mm in diameter on a 3mm thick basis and harbor a microstructure on the top. The pads of 100 µm x 100 µm are separated by trenches of 41 µm in width and 5 µm in depth to ensure liquid drainage during the contact and separation process to the lower glass slide. For the experiment, the stamps are functionalized with a 1:1 mix of NH_2-PEG-biotin (MW 3400) and NH_2-PEG-CH_3 (MW 2000; Rapp Polymere, Tübingen, Germany) and subsequently with 1mg/ml streptavidin (Thermo Fisher Scientific, Bonn, Germany) in 1xPBS containing 0.4% (w/v) BSA. Prior to the measurement, they were rinsed with 0.05% Tween 20 (VWR Scientific GmbH, Germany) in 0.2xPBS and gently dried with N_2 gas.

MFA Contact Process, Readout and Analysis

A detailed description of the measurement and analysis process of the MFA can *e.g.* be found in [1]. In short, a custom-build contact device is mounted on an inverted epi-fluorescence microscope, permitting fluorescence readout of the glass slide. A piezoelectric actuator enables the contact and separation process between slide and PDMS stamp which is controlled using reflection interference contrast microscopy [2]. The initially separated surfaces are left in contact for 10 minutes to allow for the coupling of the molecular complexes on the slide to the stamp *via* the Biotins on the uppermost DNA strand. Retraction of the stamp occurs at constant velocity of 1 µm/s. Before and after the contact of the stamp to the lower glass slide, the fluorescence intensity of the Cy5 ("RED_{Start}" and "RED_{Final}") and the FRET signal ("$FRET_{Start}$" and "$FRET_{Final}$") are recorded for each spot of molecular complexes on the slide.

In the analysis, the ratio of RED_{Final} to RED_{Start} gives the amount of intact lower bonds after stamp retraction in comparison to the initial amount of complexes: $Ratio_{RED} = RED_{Final} / RED_{Start}$. In order to correct for the complexes that have not been under load, the ratio of FRET signal is being subtracted, as a FRET signal only remains if the complexes are still fully assembled: $Ratio_{FRET} = FRET_{Final} / FRET_{Start}$. Normalization to the Coupling Efficiency CE = 1- $Ratio_{FRET}$ of complexes to the stamp yields the Normalized Fluorescence:

$NF = (Ratio_{RED} - Ratio_{FRET}) / CE$.

Hence, the NF gives the ratio between broken upper complexes in question and total amount of complexes that have been under force load. This means that the closer the NF to 0, the more stable the complex in question in comparison to the reference DNA duplex and *vice versa* for a NF closer to 1. Ideally, if the mechanical strength of both complexes is identical, the NF would be 0.5. The deviation from 0.5 in the case of the unmodified duplex against the reference of identical length and GC content can be attributed to the different positions of the GC pairs stabilizing the sequence more than AT pairs. The difference in the sequence is necessary to prevent for cross-hybridization. Additionally, the symmetry break due to the different surfaces to which the oligonucleotides are attached can play a role. The minor imbalance does not affect the result, as all samples are tested against the same reference and the effect thus cancels out.

The analysis is performed automatically using a customized LabView software which divides the original fluorescence images after background correction pixel-by-pixel according to the equation for NF and corrects for bleaching effects. The NF is then determined by fitting a Gaussian to the resulting histogram of counts.

AFM Sample Preparation

Samples for the measurement with the atomic force microscope were prepared with small changes as described previously [3]. In short, the oligonucelotides were immobilized on the amino-modified cantilever and glass surface (3-aminopropyldimethylethoxysilane; ABCR GmbH, Karlsruhe, Germany) at their 5'-termini *via* heterobifunctional NHS-PEG-Maleimide spacers (MW 5000; Rapp Polymere, Tübingen, Germany). The PEG was dissolved in a concentration of 25 mM in borate buffer at pH 8.5 and incubated for 1h. Possible disulfide bonds between oligonucleotides were reduced by TCEP incubation (Thermo Fisher Scientific, Bonn, Germany) and subsequent ethanol precipitation. The reduced DNA strands were incubated in concentrations of 5µM (surface) and 15 µM (cantilever) for 1h before a final washing step and storage in 1xPBS until use. For a parallel characterization of the individual unbinding forces in a single experiment, three distinct populations of the investigated DNA strands with propynyl modifications were incubated on one glass surface.

For all measurements, BL- AC40TS-C2 cantilevers (Olympus, Tokyo, Japan) were employed. The DNA oligomers were purchased including all modifications from biomers.net GmbH:

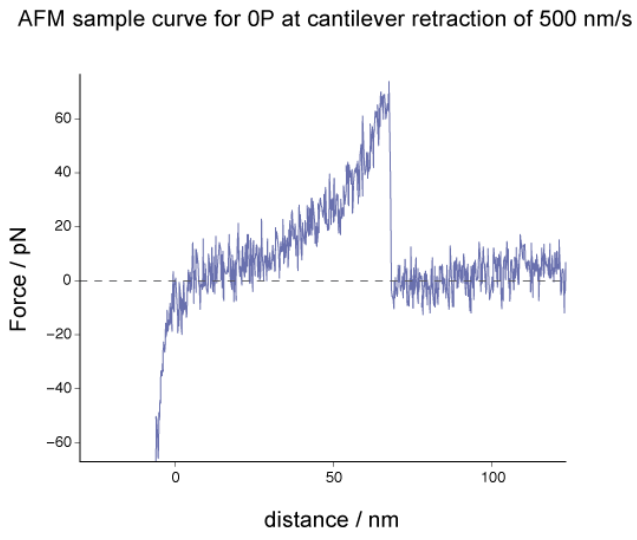
Cantilever Strand
SH - 5' - $(t)_{10}$ - tag gta gtg gag ttc aat cgg tga atc tat cag tcg tgg tgg tca gca g - 3' - **(Cy5)**
Surface Strands
(0P) SH - 5' - $(t)_{10}$ - ctg ctg a**(Cy3)**cc acc acg act gat aga ttc acc gat tga act c - 3'
(8P) SH - 5' - $(t)_{10}$ - **c**tg **c**tg a**(Cy3)**c**c** acc acg a**c**t gat aga tt**c** ac**c** gat tga a**c**t **c** - 3'
(22P) SH - 5' - $(t)_{10}$ - **ct**g **ct**g a**(Cy3)cc** a**cc** a**c**g a**ct** ga**t** aga **ttc** a**cc** ga**t** **t**ga a**ct** **c** - 3'

AFM Measurement and Analysis

Single-molecule AFM experiments were carried out on a custom built atomic force microscope, controlled by an MFP-3D controller from Asylum Research (Santa Barbara, CA, USA), which provides ACD and DAC channels as well as a DSP board for setting up feedback loops. The protocol for data recording was executed by a custom written Igor Pro (Wave Metrics, Lake Oswego, USA) software and cantilever actuation in the z direction was performed by a LISA piezo-actuator (Physik Instrumente, Karlsruhe, Germany) driven by the AFM controller. During surface approach, an indentation force of typically around 180 pN was used. The conversion from photodiode voltages into force values was performed after cantilever spring constant calibration by the thermal method using the equipartition theorem [2]. A typical spring constant in the range of 100 pN/nm and a resonance frequency of 25 kHz were obtained. After each force-extension trace the probed surface was moved by an actuated x-y stage for 100 nm to expose the DNA anchor on the cantilever to a new binding partner.
The obtained data sets for each pulling speed typically showed a yield of about 10% to 25% specific interactions of a total of 68800 curves recorded. Curves were sorted to contain exclusively single peak events with a worm-like chain behavior. The loading rate for each peak was determined as a linear fit to the in force over time in the last 4 nm before a rupture event.
Importantly, to allow for direct comparability and exclude calibration effects, the data given here have been obtained with one single cantilever. However, further experiments have reproducibly shown that the most probable rupture force cannot be distinguished for different DNA modifications in AFM experiments.

Sample AFM force-distance curve

Force-distance curves of single-binding events display a behavior that allows to preselect them using the WLC model as a criterion. However, no information is deduced from this fit. The short persistence length of 0.1-0.5 nm is a general feature of DNA measurements with AFM and consistent with previous studies. It is dominated by the very short persistence length of the PEG linkers used to attach the oligonucleotides to cantilever and surface, as they are the longest components of the system, which are stretched.

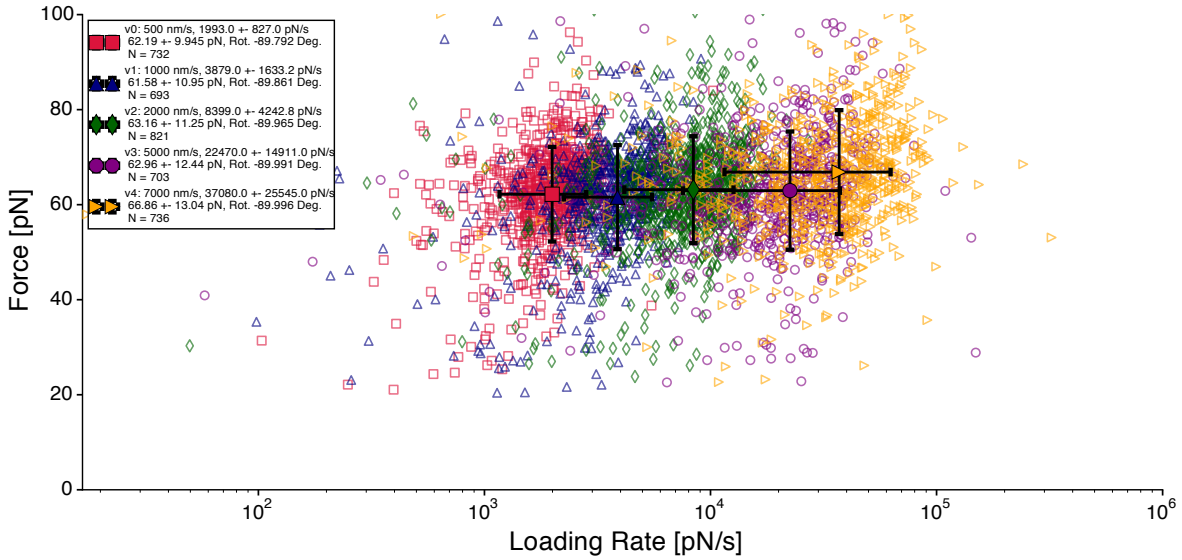


2. Supplementary Data

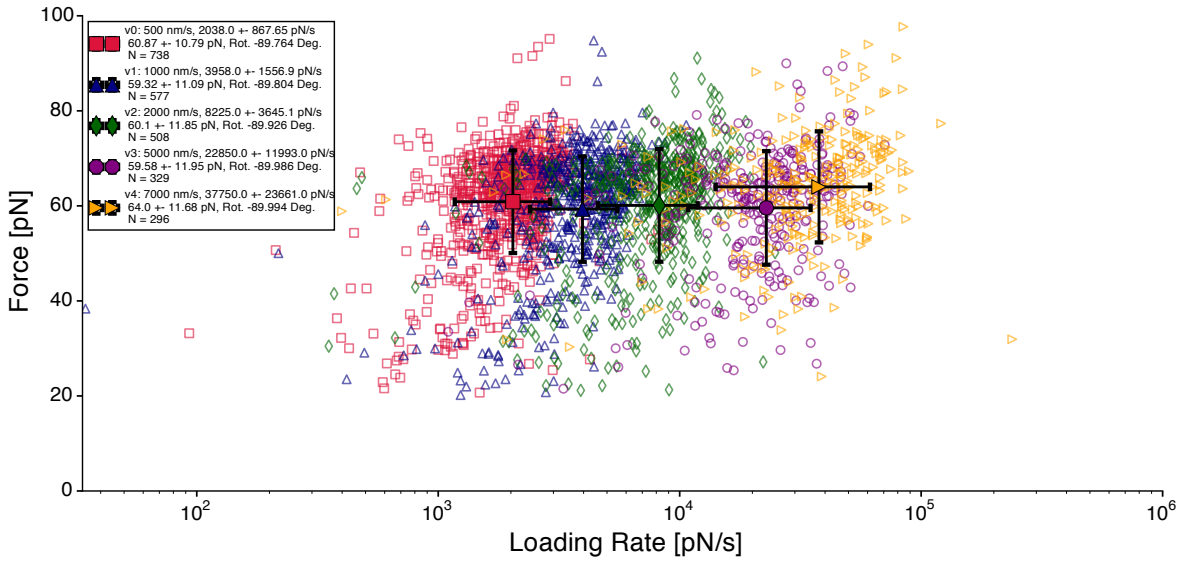
Force - Loading Rate Plots of AFM Measurements

The force-loading rate plots assembled below were fitted with an elliptical two-dimensional Gaussian to determine their respective population means and standard deviation for each retraction speed. As can be seen comparing the force-loading rate plots for 0P, 8P and 22P, the most probable rupture force for each retraction velocity are indistinguishable within error. Additionally, the rupture forces for the different retraction velocities for each variant display no significant loading rate dependence.

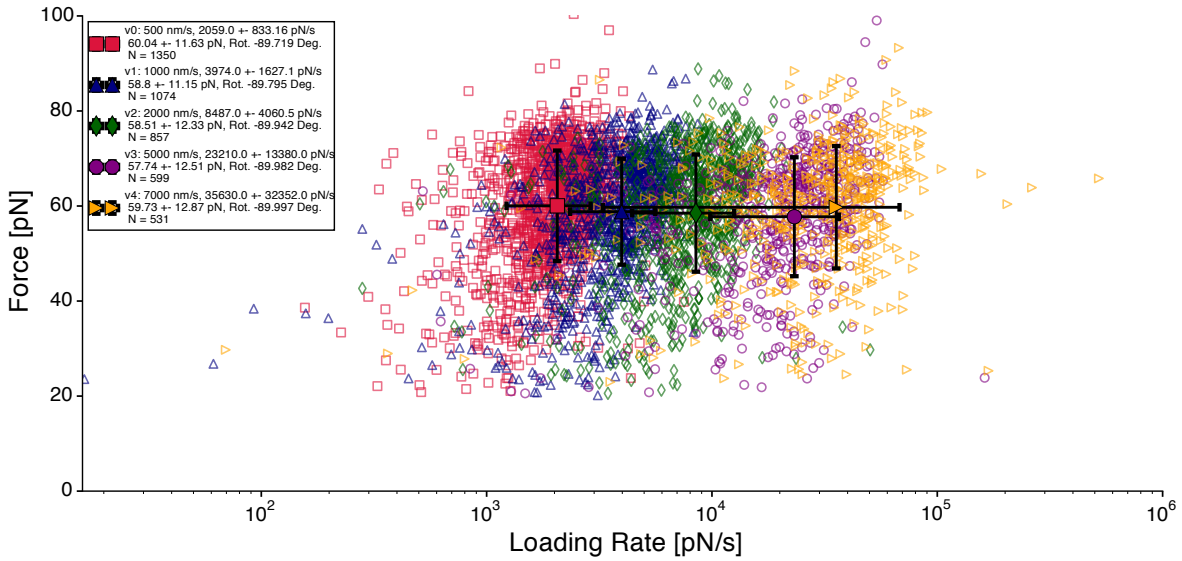
Force - Loading Rate Plot for 0P



Force - Loading Rate Plot for 8P



Force - Loading Rate Plot for 22P



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