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Probing of miniPEG γ -PNA-DNA Hybrid Duplex Stability with AFM Force Spectroscopy

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Abstract

Peptide nucleic acids (PNA) are synthetic polymers, the neutral peptide backbone of which provides elevated stability to PNA-PNA and PNA-DNA hybrid duplex. It was demonstrated that incorporation of diethylene glycol (miniPEG) at the γ position of the peptide backbone increased the thermal stability of the hybrid duplexes (Sahu, B. et al. (2011) *Journal of Organic Chemistry* 76, 5614-5627). Here, we applied atomic force microscopy (AFM) based single molecule force spectroscopy (SMFS) and dynamic force spectroscopy (DFS) to test the strength and stability of the hybrid 10 bp duplex. This hybrid duplex consisted of miniPEG γ -PNA and DNA of the same length (γ^{MP} PNA-DNA), which we compared to a DNA duplex with a homologous sequence. AFM force spectroscopy data obtained at the same conditions showed that γ^{MP} PNA-DNA hybrid is more stable than the DNA counterpart, 65 ± 15 pN vs 47 ± 15 pN, respectively. The DFS measurements performed in a range of pulling speeds analyzed in the framework of the Bell-Evans approach yielded a dissociation constant, $k_{\text{off}} \sim 0.030 \pm 0.01 \text{ sec}^{-1}$ for γ^{MP} PNA-DNA hybrid duplex vs. $0.375 \pm 0.18 \text{ sec}^{-1}$ for the DNA-DNA duplex suggesting that the hybrid duplex is much more stable. Correlating the high affinity of γ^{MP} PNA-DNA to slow dissociation kinetics is consistent with prior bulk characterization by surface plasmon resonance. Given the growing interest in γ^{MP} PNA as well as other synthetic DNA analogues, the use of single molecule experiments along with computational analysis of force spectroscopy data will provide direct characterization of various modifications as well as higher order structures such as triplexes and quadruplexes.

Peptide nucleic acid (PNA) is a synthetic analogue of DNA in which the natural negatively charged phosphodiester backbone is replaced by an artificial uncharged pseudo-peptide¹⁻³. The lack of electrostatic repulsions leads to high affinity hybridization of PNA to complementary DNA and RNA while the unnatural backbone leads to high biochemical stability to nuclease and protease enzymes. These favorable properties have led to the use of PNA in numerous applications including fluorescence *in situ* hybridization⁴, PCR clamping⁵ and regulation of gene expression⁶⁻¹⁰.

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In spite of these successes, wider adoption of PNA has been hampered by the tendency of PNA oligomers to aggregate and adsorb to surfaces. However, modification of the PNA backbone through introduction of a water-solubilizing minipolyethylene glycol (MP) substituent at the gamma carbon (γ^{MP} PNA) alleviated these technical problems¹¹. Moreover, the new chiral center at the gamma carbon pre-organizes γ^{MP} PNA, as well as other variations of γ PNA having different substituents, into a helical conformation, leading to even higher affinities than regular PNA when the appropriate configuration is used¹². This affinity is sufficiently high to allow γ^{MP} PNA to bind complementary targets even in the context of double helical DNA¹³.

The high affinity of γ^{MP} PNA was previously demonstrated by bulk methods such as UV melting curve analysis. Surface plasmon resonance analysis indicated that the high affinity, relative to standard PNA, derives from a significantly slower off-rate rather than a faster on-rate¹¹. While these methods allowed parsing of the binding improvements between association and dissociation, we were interested to study γ^{MP} PNA hybridization at the single molecule level, where individual binding and dissociation forces can be measured.

Atomic force microscopy (AFM) based single molecule force spectroscopy (SMFS) experiment can measure the force and extension involved in stretching a bond formed by intra- and inter molecular interaction¹⁴⁻¹⁶. This technique has been extensively used to study several systems including folding/unfolding of biomolecules¹⁷⁻²¹, peptide-peptide interactions²²⁻²⁴, ligand-receptor interactions²⁵⁻²⁷, and other molecular interactions²⁸. In the SMFS experiment, the molecule of interest and its target are functionalized on the probe and surface. Upon approaching the surface, the molecule tethered to the probe interacts with the target molecule attached to the surface; this interaction breaks upon retraction of the probe from the surface, producing a signature rupture force. Statistical analysis of the rupture distances and rupture forces measured can be further analyzed to study the strength and dynamics of the interaction. AFM based SMFS has been used before to study the strength and dynamics of DNA duplexes²⁹⁻³³. Recent development of the computational modeling of the AFM force spectroscopy experiment made it possible to structurally characterize the probed complex³⁴, thus opening prospects for comparative characterization of various modifications of PNA.

We have used the AFM based single molecule force spectroscopy (SMFS) and dynamic force spectroscopy (DFS) technique to measure the strength and stability of the 10 base pair (bp) γ^{MP} PNA-DNA hybrid duplex. In this approach, 10 nucleotide long γ^{MP} PNA, and a single stranded DNA (ssDNA) containing its base complement are immobilized on the mica surface and the AFM tip respectively, allowing us to study the interaction of γ^{MP} PNA with ssDNA during the multiple approach retraction cycle of the AFM tip over various location on the mica substrate. In our knowledge, this is the first SMFS and DFS study on γ^{MP} PNA-DNA hybrid duplex. We also compared the strength and stability of this hybrid duplex with a homologous DNA-DNA duplex. Our result shows that hybrid γ^{MP} PNA-DNA duplex is stronger than the DNA-DNA duplex. DFS allowed us to estimate the energy landscape and the life time associated with the rupture of a 10 bp hybrid duplex ($k_{\text{off}} = 0.030 \pm 0.01 \text{ sec}^{-1}$, $x_{\beta} = 0.63 \pm 0.02 \text{ nm}$, $G = 33 \text{ k}_B T$). Using the same experimental setup, we observed a tenfold

reduction in the strength of 10 bp DNA duplex ($k_{\text{off}} = 0.375 \pm 0.18 \text{ sec}^{-1}$, $x_{\beta} = 0.69 \pm 0.12 \text{ nm}$, $G = 30 \text{ k}_B T$).

Material and Methods

γ^{MP} PNA

The thiol-terminated γ^{MP} PNA oligomer ($\text{H}_2\text{N-GAT GGA TGA G-(PEG}_8)_2\text{-MPA}^*$, *MPA = mercapto propionic acid) was obtained from PNA Innovations, (Woburn, MA). PEG8 corresponds to an amino acid containing 8 ethylene glycol units; two PEG8 residues at the N terminus acts as a spacer which reduces non-specific interaction with the surface. All positions in the oligomer contained the γ -miniPEG modification. *Single stranded DNA (ssDNA)*. oligonucleotides were purchased from IDT (Integrated DNA Technology, Inc. Coralville, Iowa). DNA1 (30 nucleotide), was used to probe γ^{MP} PNA via the 3'- terminal nucleotides, which can form a 10 bp γ^{MP} PNA-DNA hybrid duplex. SMFS experiment on a homologous 10 bp DNA duplex was done using DNA2 (10 nt), which is complementary to the 3'-end of DNA1. 5'-end of DNA1, and DNA2 contains a "SH" group to facilitate their attachment to the AFM tip via hetero-functional poly ethylene glycol (PEG) linker with maleimide and succinimide at the ends. Here, we have studied short DNA fragments that rupture before the B-S transition occurs.

DNA1

SH-5'-TTTTTTTTTGCCTTCTACACTACCTACTC-3'

DNA2

SH-5'GAGTAG GTA G-3'

Surface chemistry

Before attachment to their respective surfaces, the γ^{MP} PNA and the ssDNA molecules were thiol-deprotected by DTT treatment. Removal of DTT from the solution was done by repeated washing with ethyl-acetate as describe in the IDT protocol. Functionalization of AFM tip with DNA1, or DNA2, and mica surface with γ^{MP} PNA was carried out according to a previously published procedure^{23, 24, 35}. The schematic in Figure 1A shows the surface chemistry involved in the attachment of the γ^{MP} PNA and the DNA1. Briefly, 200 nM γ^{MP} PNA was immobilized on an amino functionalized mica surface via hetero-functional PEG (MW = 3400 Dalton) linker with succinimide and maleimide across each end³⁶. Similar strategy was used to attach a DNA1, or DNA2 (10 μM) molecule to the AFM tip. 500 μM PEG was used to ensure a uniform coverage of the APS treated mica surface and the AFM tip.

Single molecule force spectroscopy (SMFS) and dynamic force spectroscopy (DFS)

All SMFS experiments were done on MFP3D (Oxford Instruments, Santa Barbara, CA) using silicon nitride cantilevers (MSNL, Bruker, CA) with stiffness values in the range 30-40 pN/nm. All measurements were done in HEPES buffer (10 mM HEPES, 100 mM NaCl, PH 7.0). The data analysis was performed as described^{20, 35, 37, 38}. Briefly, DFS data

was obtained for pulling experiments performed at different velocities (100-3000 nm/sec). For each pulling experiment, one thousand force curves were collected after probing at different location on the mica surface (5000 nm × 5000 nm grid). Out of this set, force curves corresponding to the characteristic rupture events were selected (a typical yield was 5%). The selection was based on the rupture location defined by the length of the PEG tether (~ 50 nm; Fig. 1A) and a non-linear extension pattern corresponding to the entropic PEG polymer extension. This part of the force curve was approximated with a worm-like chain (WLC) model as illustrated in Fig. 1B. This analysis made it possible to obtain contour length (Lc) and rupture force (F) values. The WLC fit provides the value of the persistence length of stretchable tethers³⁷. For data analysis, Igor Pro software (Asylum Research, Santa Barbara, CA) was used. Control experiments without the DNA attached were done. In these control experiments, nonlinear polymer like extension was not observed in the force curves. Also, the force values were measured in the range of instrumental noise (10-15 pN), which further show the specificity of our experimental setup in detecting γ^{MPNA} -DNA interaction.

For each force curve the apparent loading rate (ALR) was calculated using Eq. 1 and this data was used for DFS analysis. The range of apparent loading rates was divided into five groups. The force data in each group was fitted with the probability density function (PDF, Eq. 2). The values of the rupture forces corresponding to the maxima of PDF distributions were plotted against a logarithm of the mean apparent loading rate for this range using Eq. 3. From the linear DFS plots, off-rate constants (k_{off}) and the distance to the transition state (x_{β}) were calculated. Using k_{off} the barrier height (ΔG) was calculated (Eq. 4).

$$\frac{1}{r} = \frac{1}{(k_{sp}v)} \left(1 + \frac{k_{sp}L_c}{4} \sqrt{\frac{F_p}{F^3}} \right) \quad (1)$$

$$p(F) = k_{\text{off}} \exp\left(\frac{Fx_{\beta}}{k_B T}\right) \frac{1}{r} \exp\left(-k_{\text{off}} \int_0^F \exp\left(\frac{Fx_{\beta}}{k_B T}\right) \frac{1}{r} df\right) \quad (2)$$

$$F = \frac{k_B T}{x_{\beta}} \ln \left(\frac{r \cdot x_{\beta}}{K_{\text{off}} k_{\beta} T} \right) \quad (3)$$

$$\Delta G = \ln \left(\frac{k_B T}{k_{\text{off}} h} \right) k_B T \quad (4)$$

Where k_{off} is off-rate constant, F is the rupture force (RF), Lc is the contour length, $F_p = k_B T/L_p$, L_p is the persistence length, x_{β} is the distance of the transition state to the bound state, r is the apparent loading rate (ALR), k_{sp} is the spring constant of the cantilever, v is the pulling speed, k_B is the Boltzmann constant, and h is Planck's constant.

Results and Discussion

Single molecule force spectroscopy (SMFS) setup

Schematics of the SMFS setup for probing of γ^{MP} PNA-DNA hybrid duplex is shown in Figure 1A. Thiol groups at the N-terminus of the γ^{MP} PNA and the 5' end of the ssDNA were used for immobilization of interacting partners on the functionalized mica substrate and the AFM tip, respectively via maleimide terminated PEG tethers (Figure 1A). The AFM tip was brought to the mica surface and after a dwell time of 0.5 seconds, the AFM piezo was retracted by 200 nm at constant velocities in the range of 100-3000 nm/sec. Probing was done by a multiple approach-retract cycle over various spots on the mica surface and histograms for force distributions were generated. The data was approximated with the probability density functions (PDF) (Eq. 2) and the most probable rupture force that corresponded to the maximum of the PDF approximation was calculated. A similar experimental setup was used to study 10 bp DNA duplex rupture in which complementary ssDNA molecules were attached to the AFM tip and the mica surface.

Strength of γ^{MP} PNA-DNA hybrid duplex vs. DNA duplex

A typical force curve corresponding to the rupture event is shown in Fig. 1B (red). During retraction of the AFM probe, the linkers and single stranded DNA segments are extended until the bond dissociation occurs. The black line over the force curve shows the approximation of the experimental data with the worm-like chain (WLC) approximation to model the extension of flexible tethers and non-complementary ssDNA. As it is seen in Fig. 1 B, WLC works well for these experiments. Two major parameters, the rupture force and the contour length of extended flexible tethers, were calculated from this analysis. The force curve for the control experiment with no γ^{MP} PNA attached is shown above the red force curve. In the control experiment, nonlinear polymer like extension was not observed in the force curves. Also, the force values were measured in the range of instrumental noise (10-15 pN), which further shows the specificity of our experimental setup in detecting γ^{MP} PNA-DNA interaction.

Figure 2A and B shows the overlay of 40 rupture events corresponding to probing of γ^{MP} PNA-DNA hybrid and DNA homoduplex, respectively. In both datasets the rupture curves corresponding to different rupture events overlay with each other, illustrating visually the data reproducibility. The yield of interaction event was $\sim 5\%$. The major difference in the two datasets is the larger rupture force values for γ^{MP} PNA-DNA hybrid compared with those for the DNA homoduplex. The rupture force values for each experiment were measured as described in the Materials and Methods section and the histograms for the rupture force distributions for the γ^{MP} PNA-DNA hybrid and the DNA homoduplex are shown in Figs. 2C and D, respectively. The rupture force values are indeed higher for the hybrid duplex and the maxima of PDF distributions are 65 ± 15 pN and 47 ± 15 pN for γ^{MP} PNA-DNA and DNA-DNA duplexes, respectively, suggesting that γ^{MP} PNA-DNA hybrid is much stronger compared to the DNA homoduplex ($p \ll 0.001$).

Another parameter that was measured from the WLC analysis of the force curves is the contour length. The results for both systems are shown in Fig. 3. The distributions were

approximated with Gaussians and the values are 67 ± 3 nm, 66 ± 4 nm for γ^{MP} PNA-DNA hybrid and DNA homoduplex, respectively. According to the scheme in Fig. 1A, the contour length comes from the extension of two PEG tethers and ssDNA. Assuming that the length of each glycol unit in a PEG linker is equal to 0.358 nm³⁹, the length of a fully extended ssDNA is equal to 0.6 nm per nucleotide⁴⁰, and the length of a base pair is equal to 0.34 nm, then the end to end distance of a fully stretched polymer represented in (Figure 1A) is estimated to be ~ 65 nm. This value is very close to the measured L_c .

Similarity between the estimated and anticipated contour lengths provide additional validation for the experimental setup.

Stability of γ^{MP} PNA-DNA hybrid duplex vs. DNA duplex: Dynamic Force Spectroscopy studies

Next, we characterized stabilities in both duplexes using the Dynamic Force Spectroscopy (DFS) approach. According to the Bell-Evans model^{30, 32, 41, 42}, in DFS experiments probing is performed at different pulling rates (100-3000 nm/sec). According to Eq. 3, such experiments allow one to extract the system dissociation rate or the lifetime and the distance to the transition state (x_β). In this approach the rupture force values are plotted against the loading rate to calculate the necessary parameters. Given the contribution of elastic tethers to the rupture process, the DFS analysis was performed with apparent loading rates (ALR; Eq. 1). We use the approach described earlier²⁴ to arrange the datasets collected at various pulling velocities against ALR. For each group, the most probable rupture force was calculated and these values were plotted against log values of ALR (Eq. 3). The plots for both systems are shown in Figure 4. Both datasets fit well with the linear approximation, suggesting that the dissociation processes undergo the single barrier mode of the Bell-Evans model. The k_{off} of the complex and the distance of the transition state to the bound state, x_β , (see Eq. 3) were calculated from the plots. For γ^{MP} PNA-DNA hybrid duplex, $k_{\text{off}} = 0.030 \pm 0.01 \text{ sec}^{-1}$, $x_\beta = 0.63 \pm 0.02 \text{ nm}$ were obtained. For the homologous DNA duplex, under similar conditions, the Bell-Evan fit leads to $k_{\text{off}} = 0.375 \pm 0.18 \text{ sec}^{-1}$ and $x_\beta = 0.69 \pm 0.12 \text{ nm}$. The major difference is the γ^{MP} PNA-DNA hybrid has one order of magnitude higher stability than the DNA homoduplex. These results are consistent with prior surface plasmon resonance experiment, which showed significantly slower off-rates for γ^{MP} PNA compared with achiral PNA¹.

Overall, the single molecule AFM force spectroscopy studies revealed that a mini-PEG unit inserted at the γ position (γ^{MP}) of each residue in the peptide backbone leads to a dramatic stabilization of the hybrid γ^{MP} PNA-DNA duplex compared to the homoduplex. This modification increases two major parameters of the system. First, the strength of the γ^{MP} PNA-DNA duplex is increased as evidenced by the rupture force values (65 ± 15 pN vs. 44 ± 15 pN for the homoduplex). Second, the stability of the γ^{MP} PNA-DNA duplex is characterized by the lifetime being an order of magnitude higher compared with the homologous DNA duplex. A recent DFS study on 6 bp PNA-DNA hybrid duplex reported higher rupture force (~ 150 pN), with ~ 100 fold less stability compared to our hybrid duplex⁴³. Significantly larger concentration of PNA (15 μM) used in their experiment compared to 200 nM of γ^{MP} PNA used in our experiment could contribute to multiple PNA-

DNA interaction noticeable on the force extension curves. Inclusion of multiple rupture events in the DFS spectra can contribute to error prone calculation of the energy landscape ⁴¹.

The results described here suggest a number of future directions. For example, γ^{MP} PNA exhibits higher sensitivity to single mismatches than does regular PNA. Single molecule analysis could reveal insights into the origin of the improved selectivity that would be difficult to ascertain from bulk methods. Alternatively, studying more complicated structures such as a strand invasion complex in which γ^{MP} PNA is bound to double-stranded DNA ¹³ or a G quadruplex target ⁴⁴ would enhance our understanding of the rich array of hybridization modes available to this DNA analogue.

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Abbreviations

PNA	Peptide nucleic acid
mPEG	miniPEG
γ^{MP}PNA	miniPEG γ -PNA
AFM	atomic force microscopy
SMFS	single molecule force spectroscopy
DFS	dynamic force spectroscopy
PDF	probability density function

Figure 1.

(A) Schematics of the experimental setup showing DNA1 (Black line) functionalized to the AFM probe interacts with $\gamma^{\text{MP}}\text{PNA}$ (Red line) upon approaching the surface. For clarity, single $\gamma^{\text{MP}}\text{PNA}$ and DNA1 molecule tether via a PEG linker to the mica surface and the AFM tip has been shown. “S” represents thiol: maleimide interaction; (B) Force extension curves showing no interaction (black) or specific $\gamma^{\text{MP}}\text{PNA}$ -DNA interaction (Red). The WLC approximation is shown by a black line in the red rupture force curve.

Figure 2. Overlay of the FEC showing rupture of 10 bp (A) γ^{MP} PNA-DNA hybrid duplex; (B) DNA duplex. Most probable rupture force for 10 bp (C) γ^{MP} PNA-DNA hybrid duplex was calculated 65 ± 15 pN; ALR= 4641 pN/sec; (D) DNA duplex was calculated 47 ± 15 pN, ALR= 4882 pN/sec.

Figure 3.

Distributions of contour lengths for (A) γ^{MP} PNA-DNA hybrid duplex and (B) DNA duplex. The distributions were approximated with Gaussians with maxima 67 ± 3 nm and 66 ± 4 nm for PNA-DNA heteroduplex and DNA homoduplex, respectively.

Figure 4.

The dependence of rupture force values on logarithm of apparent loading rate for pulling of γ^{MP} PNA-DNA hetero duplex (squares, red; $k_{\text{off}} = 0.030 \pm 0.01 \text{ sec}^{-1}$) and 10 bp DNA homoduplex (circles, blue; $k_{\text{off}} = 0.375 \pm 0.18 \text{ sec}^{-1}$). Error bars represent standard error of the mean.