Interaction of the Hydrophobic Tip of an Atomic Force Microscope with Oligopeptides Immobilized Using Short and Long Tethers

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ABSTRACT: We report an investigation of the adhesive force generated between the hydrophobic tip of an atomic force microscope (AFM) and surfaces presenting oligopeptides immobilized using either short (~1 nm) or long (~60 nm) tethers. Specifically, we used either sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) or 10 kDa polyethylene glycol (PEG) end-functionalized with maleimide and N-hydroxysuccinimide groups to immobilize helical oligomers of β-amino acids (β-peptides) to mixed monolayers presenting tetraethylene glycol (EG4) and amine-terminated EG4 (EG4N) groups. When SSMCC was used to immobilize the β-peptides, we measured the adhesive interaction between the AFM tip and surface to rupture through a single event with magnitude consistent with the interaction of a single β-peptide with the AFM tip. Surprisingly, this occurred even when, on average, multiple β-peptides were located within the interaction area between the AFM tip and surface. In contrast, when using the long 10 kDa PEG tether, we observed the magnitude of the adhesive interaction as well as the dynamics of the rupture events to unmask the presence of the multiple β-peptides within the interaction area. To provide insight into these observations, we formulated a simple mechanical model of the interaction of the AFM tip with the immobilized β-peptides and used the model to demonstrate that adhesion measurements performed using short tethers (but not long tethers) are dominated by the interaction of single β-peptides because (i) the mechanical properties of the short tether are highly nonlinear, thus causing one β-peptide to dominate the adhesion force at the point of rupture, and (ii) the AFM cantilever is mechanically unstable following the rupture of the adhesive interaction with a single β-peptide. Overall, our study reveals that short tethers offer the basis of an approach that facilitates measurement of adhesive interactions with single molecules presented at surfaces.

INTRODUCTION

Quantification of the adhesive interaction of a chemically functionalized tip of an atomic force microscope (AFM) with a surface decorated with binding groups (e.g., immobilized oligopeptides, proteins, or colloids) has been widely used to provide insight into the origins of interfacial interactions, including hydrophobic and/or hydrogen bond-mediated interactions.†−§ Of particular relevance to the study described in this Article, we recently reported characterization of water-mediated interaction of a hydrophobic AFM tip with oligomers of β-amino acids (β-peptides) immobilized at surfaces.⁶,⁷ While these past measurements of adhesive (pull-off) forces were found to depend on the chemical nanopattern encoded by the oligopeptides, a surprising observation, which is explored further in the study described herein, was that forces of a magnitude consistent with single β-peptide interactions were measured under conditions for which multiple β-peptides were expected to be within the contact area formed between the AFM tip and the surface presenting the β-peptides.⁶,⁷ Building from this observation, we explore further conditions under which such single-molecule interactions can be measured at surfaces, a result that we judge to be broadly relevant and useful to AFM-based methods for characterization of intermolecular forces.

The interaction of an AFM tip with a surface is generally interpreted to correspond to a single-molecule event when (i) the magnitude of the pull-force falls within a range typical of single-molecule interactions (~0.01−1 nN, depending on the type of interaction),¹,²,⁵ (ii) the majority of interactions between the AFM tip and surface do not result in adhesion, thus indicating that the density of adhesive molecules on the surface is sufficiently low (see below for a quantitative statement) that the probability of multiple adhesive molecules interacting simultaneously with the AFM tip is vanishingly small,³−⁶,⁸−¹¹ and (iii) the AFM tip exhibits a single “jump”, upon retraction from the surface, corresponding to the rupture of a single (physical) bond between the AFM tip and the surface.¹²−¹⁵ Although these criteria have been employed widely in past studies, as detailed below, we interpret AFM force measurements involving surface immobilized β-peptides to correspond to single β-peptide events when performed...
under conditions that do not satisfy the above-stated criteria. In the remainder of this introduction, we discuss each of the above-stated criteria as we return to them when interpreting our experimental measurements.

As noted above, AFM pull-off measurements obtained when the majority of tip–sample contacts do not result in an adhesive event are often interpreted to correspond to single-molecule interactions.\textsuperscript{1,3,8,11} Under these conditions, the interaction of the AFM tip with the surface is generally assumed to follow Poisson statistics:\textsuperscript{19–26}

\[
P(N_b) = \frac{N_b^N e^{-\lambda}}{N_b!}
\]

where \(P(N_b)\) is the probability of forming \(N_b\) intermolecular bonds and \(\lambda\) represents the mean number of intermolecular bonds formed, which can be evaluated as\textsuperscript{22}

\[
\lambda = -\ln P(N_b = 0)
\]

where \(P(N_b = 0)\) is the frequency of nonadhesive events measured.\textsuperscript{22} By combining eqs 1 and 2, it can be calculated that if 10% of tip–sample interactions result in adhesion, there is a 95% probability that a given adhesive event will be caused by the interaction of a single binding group with the AFM tip (i.e., <5% chance that an adhesion event results from multiple binding interactions).

In addition to the fraction of nonadhesive events, the observation of a single rupture event in an AFM pull-off curve is commonly cited as evidence of a single-molecule interaction. This criterion, however, assumes that multiple binding interactions, if present between the AFM and sample surface, lead to sequential rupture events during the retraction of the AFM tip from the surface.\textsuperscript{1,8,12–18} As discussed below, whether or not single or multiple rupture events are measured during retraction of the AFM tip from the surface depends strongly on the mechanics of the interaction, as determined by the properties (including length and mechanical properties) of the tether used to immobilize the binding groups in addition to the stiffness of the AFM tip cantilever.\textsuperscript{24}

The adhesion measurements reported in this Article involve a hydrophobic AFM tip interacting with \(\beta\)-peptides comprised of trans-2-aminocyclohexanecarboxylic acid (ACHC) and \(\beta\)-homolysine (\(\beta\)-hLys) immobilized at surfaces. The \(\beta\)-peptides used in our study contain 6 ACHC residues (Figure 1A) and fold into a stable, helical secondary structure (14-helix in aqueous and alcoholic solutions from pH 2 to 12) that generates a well-defined three-dimensional chemical pattern.\textsuperscript{27–31} These oligopeptides thus make possible a systematic study of the effects of nanopatterns of charged (\(\beta\)-hLys) and hydrophobic (ACHC) residues on intermolecular interactions.\textsuperscript{6,7} Specifically, we focus on an oligopeptide that generates a nonglobally amphiphilic display of charged and hydrophobic groups (Figure 1A). Our past studies have established that this nonglobally amphiphilic oligopeptide adheres to hydrophobic AFM tips via a mode of interaction that is dominated by the charged \(\beta\)-hLys residues.\textsuperscript{6,7} Because, for reasons detailed below, we interpret our measurements of the interaction of the hydrophobic AFM tip and immobilized \(\beta\)-peptides to correspond to single-molecule forces under conditions for which the percentage of nonadhesive interactions is not low (>10%),\textsuperscript{6} we critically assess the significance of low binding probabilities in the measurement of single-molecule interactions. To this end, we report measurements of the influence of \(\beta\)-peptide surface immobilization density, and AFM tip to surface interaction time on the interactions of dodecanethiol-functionalized AFM tips with \(\beta\)-peptides (Figure 1A) immobilized at surfaces (Figure 1B). The length-dependent mechanical properties of the \(\beta\)-peptide tethers were also investigated, by using short (<10 nm) and long (>10 nm) tethers to immobilize the \(\beta\)-peptides, to determine the role of tether length on the measurement of single and multiple binding interactions.
EXPERIMENTAL SECTION

Materials. Tetra-ethylene glycol thiols terminated in hydroxyl (EG4) or amine groups (EG4N) were purchased from Prochimia (Poland). 1-Dodecanethiol (98%) and triethanolamine (TEA) HCl (99%) were purchased from Aldrich (Milwaukee, WI). 11-Aminoundecanethiol was purchased from Dojindo Molecular Technologies. Sulfofuocinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid (SSMCC) was purchased from Pierce Biotechnology (Rockford, IL.). 10 kDa heterobifunctional PEG linkers (MAL-PEG-SCM) were purchased from Creative PEGWorks. Ethanol (reagent, anhydrous, denatured) used for preparation of thiol solutions and sodium chloride (99.0%) were purchased from Sigma-Aldrich (Milwaukee, WI). Ethanol (anhydrous, 200 proof) used for rinsing was purchased from Decon Laboratories (King of Prussia, PA). Deionized water used in this study had a resistivity of 18.2 MΩ cm. All chemicals were used as received and without any further purification. The AFM tips used in this study (triangular shaped with radius of curvature of 10 nm) were purchased from Bruker (Camarillo, CA). Silicon wafers were purchased from Silicon Sense (Nashua, NH).

Preparation of β-Peptide Decorated Surfaces. β-Peptide oligomers were synthesized via solid-phase methods as described elsewhere.6 To tether β-peptides with 10 kDa heterobifunctional PEG linkers, after rinsing the EG4N/EG4 monolayers as described elsewhere,7 the substrates were incubated with the PEG linkers (50 mg/mL solution in TEA at pH 7) at room temperature for 6 h and then rinsed with deionized water, ethanol, and dried with nitrogen. β-Peptides were then immobilized on the surface as detailed previously.6,7 AFM images of bare gold films and β-peptides immobilized on EG4-terminated SAMs are shown in Figure S1.

Preparation of Mixed Surfaces. Mixed monolayers were prepared by immersing small pieces of gold-coated silicon wafers into ethanolic solutions containing 0.4 mM of 11-aminoundecanethiol and 0.6 mM decanethiol incubated for 18 h. Upon removal from solution, substrates were rinsed thoroughly with ethanol and water, dried with nitrogen, and stored in TEA buffer (10 mM, pH 7) until AFM adhesion measurements were performed. The compositions of mixed SAMs containing 40% amine-terminated undecanethiol and 60% decanethiol were determined by using X-ray photoelectron spectroscopy (XPS; see Figure S2).

Preparation of Hydrophobically Functionalized AFM Tips. Triangular-shaped cantilevers with nominal spring constants of 0.01, 0.03, and 0.1 N/m were used. AFM tips were coated with a 2 nm layer of titanium and a 20 nm layer of gold. Following gold deposition, the tips were immersed in a 1 mM ethanolic solution of 1-dodecanethiol and incubated overnight. Upon removal from the solution, the tips were rinsed with ethanol, dried with a gentle stream of nitrogen, and immediately transferred to the AFM fluid cell. After functionalization, the spring constants of the cantilevers were measured using the thermal tuning method on a Nanoscope V Multimode AFM and determined to be 0.028 ± 0.001 N/m (nominal 0.01 N/m), 0.078 ± 0.005 N/m (nominal 0.03 N/m), and 0.262 ± 0.009 N/m (nominal 0.01 N/m).

AFM Force Measurements. Adhesion force measurements were performed using a Nanoscope IIIa Multimode AFM equipped with a fluid cell (Veeco Metrology Group, Santa Barbara, CA). Triangular-shaped silicon nitride cantilevers were used and functionalized as described above. Force measurements were performed in aqueous TEA (10 mM, pH 7) at room temperature. Force curves were recorded using retraction and approach speeds of 1000 nm/s, with an applied AFM tip to surface contact force of 0.56 nN. The standard deviation and the standard error of the mean reported below were calculated from measurements performed with multiple tips and multiple substrates.

RESULTS AND DISCUSSION

Influence of β-Peptide Immobilization Density. Our initial experiments determined how the density of β-peptides immobilized on a surface influences the distribution of forces measured between a hydrophobic AFM tip and the β-peptides (including the percentage of nonadhesive tip—sample interactions). For these initial measurements, we immobilized the β-peptides using the heterobifunctional cross-linker sulfofuocinimidyl-4-((N-maleimidomethyl)cyclohexane-1-carboxylic acid (SSMCC, Figure 1B), as detailed in our past work.57 In brief, the density of immobilized β-peptide was varied by changing the mole fraction of amine-terminated EG4 (EG4N) thiol present within a mixed monolayer formed from EG4N- and EG4-terminated thiols (SSMCC reacts with the amine-terminal group of the EG4N component of the mixed monolayer). The AFM tip was made hydrophobic by coating the tip with a 20 nm thick film of gold and subsequently reacting the gold-coated tip with dodecanethiol. All measurements were performed using aqueous triethanolamine (TEA) buffer at pH 7. We comment here that the estimated length of the SSMCC tether is ~1 nm (ref 6), and thus we use it as a short tether. Additionally, we note that we demonstrated previously that SSMCC tethers attached to the above-described mixed monolayers do not generate adhesive interactions with the hydrophobic AFM tip (in the absence of the β-peptides).6 In our initial measurements, the spring constant of the AFM tip was 0.028 N/m, and the adhesive force between the AFM tip and immobilized β-peptides was quantified immediately following contact of the AFM tip with the surface (additional measurements during which the tip was equilibrated against the surface for increasing lengths of time are reported below).

Figure 1C shows how the density of β-peptides immobilized using SSMCC impacts the mean adhesive forces measured between the hydrophobic AFM tip and the β-peptide-decorated surface. Inspection of Figure 1C reveals that the magnitude of the mean adhesive force increases with surface immobilization density when the percentage of EG4N within the mixed SAM is >0.2%. In contrast, when the percentage of EG4N within the mixed SAM is ≤0.2%, the mean adhesion force (0.39 ± 0.03 nN, standard error of the mean of all samples immobilized at 0.01%, 0.1%, and 0.2% EG4N) was found to be independent of immobilization density (we note that no adhesion was measured for 0.0% EG4N). From this result, we infer that adhesive interactions between the AFM tip with the surfaces containing ≤0.2% EG4N are dominated by binding events involving single β-peptides on the surface, whereas adhesive interactions measured using surfaces containing >0.2% EG4N involve multiple β-peptides interacting simultaneously with the AFM tip.

Additional initial support for the above-stated interpretation of the measurements in Figure 1C comes from several observations. First, the magnitudes of the adhesive forces that we measure using surfaces containing ≤0.2% EG4N (0.39 ± 0.03 nN) are generally consistent with prior studies of single-molecule interactions measured with AFM.1,2,5 Second, we compare the results in Figure 1C to the mean adhesive force measured between mixed monolayers (containing 40% 11-aminoundecanethiol and 60% decanethiol) and a hydrophobic AFM tip (10.7 ± 0.5 nN, standard error of the mean of five independent samples, Figure 2). To compare the two measurements, we normalized the adhesion forces by the contact areas formed between the AFM tip and sample. We used Johnson–Kendall–Roberts (JKR) theory to estimate the radius of this contact area (a) for the mixed monolayers as:

\[
a^3 = \frac{F_{ad} R}{K}
\]  

(3)
This leads us to estimate the adhesive force per unit area gives rise to the adhesion force was calculated to be 62 nN/m². This value that is comparable to that calculated from the AFM tip radius (R = 53 ± 5 nm, Figure 3). From eq 3, the area of contact between the AFM tip and amine-terminated SAMs that gives rise to the adhesion force was calculated to be 62 ± 4 nm². This leads us to estimate the adhesive force per unit area of the amine-terminated SAM to be 0.17 ± 0.01 nN/nm², a value that is comparable to that calculated from the β-peptide force measurements assuming a single β-peptide interacts with the AFM tip (0.39 ± 0.03 nN using an estimated β-peptide molecular area of ~1 nm²). We interpret the results above to suggest that adhesion between the AFM tip and β-peptide-decorated surfaces is dominated by single β-peptide interactions when mixed SAMs containing 0.1% EG4N are used to immobilize the β-peptides. We thus performed a number of repeat measurements using independently prepared SAMs containing 0.1% EG4N and recorded the magnitude of the adhesion interaction as well as the fraction of tip-sample interactions that did not result in an adhesive event (so-called nonadhesive contacts) (Figure 4B–D). Inspection of Figure 4B–D reveals the three histograms to be similar when comparing the means and standard deviations (SD). Significantly, however, the percentage of tip-sample interactions that resulted in nonadhesive contacts varied widely between samples (from 48% to 95%). Furthermore, if we apply the commonly used criterion for single-molecule interactions at a surface, that the percentage of tip-sample interactions that result in adhesion should be ≤10% (see eqs 1 and 2 along with associated text), only the sample shown in Figure 4B would be concluded to correspond to single β-peptide interactions. We emphasize, however, that the distribution and magnitude of the adhesive forces in Figure 4C and D are indistinguishable from Figure 4B (see insets of each histogram), suggesting that the interactions shown in Figure 4C and D also likely arise from the interactions of the AFM tip with single β-peptides. These observations, and others reported below, led us to hypothesize that, for our experimental system, the interactions of single β-peptides with the AFM tip can be measured under conditions where the widely used criterion for measurement of single-molecule interactions fails (i.e., that the percentage of sample-tip interactions that result in adhesion can be >10%). We comment here that the experimentally measured sample-to-sample variation in the percentage of nonadhesive events shown in Figure 4 likely arises from differences in the AFM tip geometry used in each of our experiments as well as variations in the extent of the reactions that lead to the immobilization of the β-peptides onto the mixed SAMs.

**Influence of Tip–Sample Contact Time.** The results presented above suggest that, for our experimental system, the percentage of nonadhesive interactions does not need to be ≤10% to measure forces that arise from single β-peptide interactions with the AFM tip. This suggestion is surprising because, as noted in the Introduction, simple models of the interaction of an AFM tip with a surface predict that the adhesive forces observed in histograms with small percentages of nonadhesive interactions arise, in part, from multiple binding interactions (Poisson distribution, eqs 1 and 2). To provide additional insight into our measurements, we explored an additional means by which to vary the percentage of nonadhesive tip-sample interactions. Past studies have demonstrated that the percentage of tip-sample interactions that result in an adhesive event can be influenced by the time of contact between the AFM tip and the surface. The rise in percentage of adhesive interactions is generally attributed to the dynamics of bond formation (e.g., reorientation of the bound species). Figure 5 shows the influence of changing...
Estimation of the Number of β-Peptides Interacting with the AFM Tip. We determined the effective contact area between the AFM tip and the β-peptide-decorated surfaces to enable an estimate of the number of β-peptides interacting with the AFM tip. Initially, we based our calculation on a simple physical model that neglected the length of the SSMCC unit used to tether the β-peptides to the surface. We used Hertz theory to estimate the radius of the contact area between the AFM tip and mixed EG4N/EG4 SAM as

$$a^3 = \frac{F_{\text{applied}} R}{K}$$

(4)

where $F_{\text{applied}}$ is the force applied to the surface by the AFM tip ($F_{\text{applied}} = 0.56 \text{ nN}$; this force corresponds to the threshold force at which the approach of the AFM tip toward the β-peptide-decorated surface was stopped in our experiments), $K$ is the elastic modulus of the contacting surfaces ($K \approx 6.4 \text{ GPa}$), and $R$ is the AFM tip radius ($R = 53 \pm 5 \text{ nm}$, Figure 3). From eq 4, we estimated the contact area between the AFM tip and the mixed EG4N/EG4 SAM to be 8.8 ± 0.6 nm². Past studies have established the density of thiols within a SAM to be 1 thiol/0.214 nm². Accordingly, we estimated the surface density of β-peptides immobilized to a mixed SAM containing 0.1% EG4N to be 1 β-peptide/214 nm². Thus, for a mixed SAM containing 0.1% EG4N, the probability of finding a β-peptide with an area of 8.8 ± 0.6 nm² is (8.8 ± 0.6)/214 = 0.04 ± 0.003. If this physical scenario were correct, only 4 ± 0.3% of the tip--sample interactions would result in an adhesive binding event between a β-peptide and the AFM tip. In contrast, in our experimental measurements, we found that up to 69% of tip--sample interactions resulted in adhesion (Figures 4 and 5). From this analysis, we conclude that our measurements likely involve β-peptide--AFM tip interactions that occur outside of the above-calculated Hertzian contact area.

The simple model described above does not consider the finite length of the SSMCC tether on the interactions of the β-peptides with the AFM tip. Specifically, as shown in Figure 6,
where $y$ is the combined length of the SSMC tether and $\beta$-peptide ($y \approx 2$ nm for SSMCC-tethered $\beta$-peptides), $\delta$ is the depth of deformation of the sample according to the Hertz theory:

$$
\delta = \left( \frac{F_{\text{applied}}^2}{RK^2} \right)^{1/3}
$$

By combining eqs 5 and 6, we calculated the effective "interaction area" between the AFM tip and the $\beta$-peptide-decorated surface to be $662 \pm 64$ nm$^2$. This result, when compared to our estimate of the areal density of $\beta$-peptides immobilized on the SAM (1 $\beta$-peptide/214 nm$^2$; see above), leads us to conclude that the effective interaction area between the AFM tip and the $\beta$-peptide-decorated surface will present, on average, several $\beta$-peptides that could potentially interact with the AFM tip. This result is consistent with our experimental observation that the percentage of nonadhesive interactions is small for some of our samples (e.g., 31% for the sample in Figure 5D). Significant, however, even though multiple $\beta$-peptides are present within the interaction area between the AFM tip and sample in our experiments, we emphasize again that we interpret the magnitude of the adhesion force to be close to that of a single $\beta$-peptide (Figures 4 and 5). Additionally, we note that the pull-off curves for the SSMCC-tethered $\beta$-peptides exhibit only one rupture event (Figure 4A).

The results described above lead to two key questions: (i) why are multiple rupture events not observed during the pull-off of the AFM tip from the $\beta$-peptide-decorated surface, and (ii) why is the magnitude of the adhesion force close to that of a single $\beta$-peptide interaction when, on average, more than one $\beta$-peptide interacts with the AFM tip? Below we present experiments and analyses that address these two questions.

Why Are Multiple Rupture Events Not Observed with SSMC-Tethered $\beta$-Peptides?

We begin our discussion of this question by noting that Karacsony and Akhremitchev recently demonstrated that adhesion between an AFM tip and a surface presenting multiple binding groups can rupture as either a single event or multiple events depending on the magnitude of the AFM cantilever spring constant and the mechanical properties of the tether used to immobilize the binding groups. Specifically, when two binding groups interact with an AFM tip via two long (>10 nm) tethers that differ slightly in contour lengths ($L_c$), they concluded that two rupture events will be measured only if the spring constants ($k_c$) of the AFM cantilever exceed a critical value ($k_c$):

$$
k > k_c \propto \frac{F_{\text{rupt},1}}{L_c}
$$

where $F_{\text{rupt},1}$ is the force required to break a single bond. Notably, when the two binding groups are loaded via long (>10 nm) linkers ($F_1$ and $F_2$ in Figure 7), the initial rupture event, with a total rupture force ($F_{\text{rupt},T}$) arising from contributions of both binding pairs, occurs when the more highly loaded bond breaks ($F_1$ in Figure 7). Upon breaking of the $F_1$ bond, the AFM tip relaxes according to $k$, while the $F_2$ bond continues to be loaded according to the tether mechanics. If the AFM tip relaxes to an equilibrium position and associated force ($F_{eq}$) $< F_{\text{rupt},1}$ prior to the $F_2$ bond being loaded to rupture ($k > k_c$), a second bond rupture event will be observed when the $F_2$ bond is ultimately loaded to failure. However, if the AFM tip does not reach an equilibrium position with $F_{eq} < F_{\text{rupt},1}$ prior to rupture of the $F_2$ bond ($k \leq k_c$), the second rupture event will not be seen (Figure 7a).

Guided by this past study, we hypothesized that the AFM tips used in our experiment, with cantilever spring constants of 0.028 N/m, were too soft to permit observation of multiple bond rupture events with the $\beta$-peptides immobilized via the (short) SSMCC tethers. With reference to eq 7, we emphasize that the threshold $k_c$ needed to observe multiple bond rupture events during pull-off increases with decreasing tether length ($L_c$). Therefore, in an effort to reveal the multiple $\beta$-peptide interactions in our experiments that we hypothesized to be "hidden" by the short SSMCC linker, we made three changes to the design of our experimental system: (i) we increased the length of the tether by using the 10 kDa heterobifunctional polyethylene glycol (PEG) linker (longest commercially available PEG linker with maleimide and N-hydroxysuccinimide end groups) to immobilize the $\beta$-peptides, (ii) we increased the stiffness of the AFM cantilevers used in our experiments from 0.028 to 0.26 N/m, and (iii) we contacted the AFM tip with the surface presenting the immobilized $\beta$-peptide for 500 ms to increase the probability of forming multiple bonds. Lc of the 10 kDa PEG tether was estimated to be 60 nm (for PEG in an aqueous environment; see the Supporting Information).
Prior to immobilizing the β-peptides with the 10 kDa PEG tether, we confirmed that the PEG tether alone, when immobilized to a 0.1% EG4N SAM, did not generate adhesive interactions between the AFM tip and the surfaces (Figure 8A).

In contrast, with β-peptides immobilized via the long PEG linker, we measured a broad distribution of adhesive forces that extended to ~1.0 nN (Figure 8B). Figure 8C reveals that rupture events measured with the long PEG linker took place at AFM tip-to-surface separation distances centered at approximately 51 nm, consistent with the loading of the β-peptides via extension of the long PEG linker (see the Supporting Information for a discussion of the rupture length distribution). In contrast to Figure 8B, adhesion forces greater than ~0.2 nN were not measured when we used the short SSMCC linkers to immobilize the β-peptides at surfaces prepared using 0.1% EG4N (Figures 4 and 5). Therefore, we interpret the larger adhesion forces (>~0.2 nN) measured with the 10 kDa PEG linkers to arise from interactions of multiple β-peptides with the AFM tip. In support of this interpretation, we observed some of the pull-off curves associated with adhesion forces of ~1.0 nN to manifest signatures of multiple rupture events (Figure 8F). In contrast, single rupture events were always observed when the adhesion force was measured to be ~0.4 nN (Figure 8E). When combined, these results support our hypothesis that multiple bond rupture events are not evident in our measurements with SSMCC-tethered β-peptides because the mechanics of the AFM tip cantilever/short tether interaction (cantilever is too soft; tether is too short) do not permit resolution of single β-peptide–tip rupture events from multiple β-peptide–tip bond ruptures (see eq 7).

Additional support for the above-described conclusion was obtained by analyzing the distance-dependence of the force applied to the AFM tip during the retraction of the AFM tip from the surface (when using the 10 kDa PEG linker). This force is transmitted to the AFM tip by the polymeric tether and thus reflects the mechanical properties of the polymeric tethers. According to the worm-like-chain (WLC) model, the force ($F(z)$) required to separate the two ends of a polymer chain by a distance $z$ is given as:

$$F(z) = k_B T \left( \frac{1}{l_p} \left( 1 - \frac{z}{L_c} \right)^{2} + \frac{z}{L_c} - \frac{1}{4} \right)$$

where $l_p$ is the persistence length of the polymer chain, $k_B$ is the Boltzmann constant, and $T$ is the temperature. By modeling the PEG linker using eq 8 and setting $F(z) = 0.34$ nN (i.e., $F_{upp}$), corresponding to the rupture of a single β-peptide binding event as determined from the mean adhesion force measured in Figures 1C, 4, and 5, $z = 51$ nm (average rupture length observed in Figure 8C), and $L_c = 60$ nm (for the 10 kDa PEG linker in an aqueous environment; see the Supporting Information), we calculated the PEG chain to have a persistence length of 0.34 nm. This value lies within the range of previously reported persistence lengths for PEG (0.28–0.35 nm), and thus confirms that a single PEG chain is transmitting a force of the magnitude (0.34 nN) that triggers detachment of single β-peptides from the AFM tip. Furthermore, as shown in Figure 8E, we were able to fit eq 8 to the pull-off curves obtained when measuring an adhesion force of ~0.4 nN by using $L_c$ and $l_p$ as adjustable parameters. This yielded an estimate of $l_p \cong 0.3$ nm. In contrast, the pull-off curves obtained when measuring adhesion forces of ~0.4 nN, with either one (Figure 8D) or two (Figure 8F) rupture events, yielded a best-fit value of $l_p \cong 0.2$ nm. In the latter scenario, the smaller $l_p$ value is consistent with past observations that the simultaneous stretching of multiple polymer chains reduces the effective $l_p$ required to describe the mechanical properties of the system.

Why Do Adhesion Forces Measured with SSMCC-Tethered β-Peptides Correlate to the Magnitudes of Single β-Peptide Interactions When, on Average, More Than One β-Peptide Interacts with the AFM Tip? The discussion above provides an explanation for the absence of multiple rupture events in our measurements with (short) SSMCC linkers, but the preceding discussion does not explain why the magnitude of the rupture force, when measured using SSMCC-tethered β-peptides immobilized to mixed SAMs containing 0.1% EG4N, is not greater than the single β-peptide rupture force (Figure 7). To address this unresolved issue, we hypothesized that the mechanical properties of short tethers...
(e.g., SSMCC) generate adhesion forces \( (F_{\text{rupt,T}}) \) that are dominated by the interaction of a single \( \beta \)-peptide (i.e., the forces generated by other \( \beta \)-peptides bound to the AFM tip are negligible). Specifically, because the geometry of the AFM tip is curved, it appeared likely to us that the degree of extension of the SSMCC tethers would vary between or among the \( \beta \)-peptides that interact with the AFM tip as the AFM tip retracts from the surface (Figure 9A).

To explore this hypothesis, we again resorted to use of the WLC model for the tether, as described by eq 8. According to the WLC model, for \( z \ll 0.4L_c \), the tether follows Hooke’s law:

\[
F(z) = k_B T \left( \frac{z}{L_c} \right)
\]

Beyond this threshold, the force–extension relationship deviates significantly from Hooke’s law with the force growing more rapidly with extension of the polymer, \( (1 - \frac{z}{L_c})^{-2} \). To explore the consequences of this deviation from Hooke’s law, we calculated the mechanical interaction of an AFM tip with two \( \beta \)-peptides bonded to locations on the AFM tip that were separated by a vertical displacement \( (H) \) (Figure 9A). Thus, for two identical linkers, the degrees of extension of the two tethers will differ by

\[
\delta H = |H_2 - H_1| > 0
\]

We analyzed the simultaneous stretching of two identical WLCs using \( \delta H = 0.5 \) nm and \( F_{\text{rupt,1}} = 0.34 \) nN (single \( \beta \)-peptide rupture force as determined from Figures 1C, 4, and 5) using either long \( (L_c > 10 \) nm) or short \( (L_c = 1 \) nm for SSMCC) linkers (Figure 9B and C). Our model differs from that used recently by Karasny and Akremitchev, who employed the freely jointed chain (FJC) model and analyzed long \( (L_c > 10 \) nm) tethers with \( \delta H = 0 \) nm. The differences in tether extension used in their calculations resulted from linker polydispersities. In contrast, our model describes monodisperse tethers, and the position of attachment to the AFM tip (defined by eq 10) leads to different extents of extension.

Figure 9B indicates that when short linkers \( (L_c = 1 \) nm) are used and the total force transmitted to the AFM tip rises to \( F_{\text{rupt,T}} \), \( F_1 \) has not deviated significantly from the Hookean force–extension regime, but \( F_2 \) is in the regime where the force transmitted by the tether scales as \( (1 - \frac{z}{L_c})^{-2} \). Thus, although
observations with SSMCC-tethered β-SSMCC-tethered multiple binding interactions. In striking contrast, when long linkers ($L_c = 60$ nm) are used, at $F_{rup,T}$, $F_1$ and $F_2$ are in similar force-extension regimes; thus $F_{rup,T}$ arises from approximately equal contributions from both $F_1$ and $F_2$ ($F_1$ represents $53\%$ of $F_{rup,T}$, Figure 9C). These results demonstrate that, when two identically tethered bonds are simultaneously loaded, small differences in bond attachment locations on the AFM tip (defined by $\delta H$) lead to distinct outcomes when using short (e.g., SSMCC) versus long (e.g., 10 kDa PEG) linkers.

Evaluating our model for values of $L_c$ between 1 and 60 nm (Figure 9E) reveals that long linkers ($L_c > 10$ nm) mask small differences in bond attachment locations on the AFM tip ($\delta H = 0.5$ nm) and thus lead to values of $F_{rup,T}$ that reflect the contributions of multiple bonds (two bonds as shown in Figure 9E). In contrast, when tether lengths are shorter than 10 nm, the tip detaches from the surface when the most highly loaded single bond breaks (i.e., $F_{rup,T} \rightarrow F_{rup,1}$ as $L_c \rightarrow 0$). Overall, this simple mechanical model supports our interpretation of the experimental results, that short tethers facilitate the measurement of single-molecule binding forces in the presence of multiple binding interactions.

Under our experimental conditions ($k = 0.028$ N/m for SSMCC-tethered β-peptides and $k = 0.26$ N/m for 10 kDa PEG-tethered β-peptides), our model predicts that multiple rupture events cannot be resolved when the tethered β-peptides are bound to the AFM tip at $\delta H = 0.5$ nm (Figure 9B and C). Although this prediction is consistent with our experimental observations with SSMCC-tether β-peptides (Figure 4A), when using 10 kDa PEG-tethered β-peptides, we do observe multiple rupture events using AFM tip cantilevers with $k = 0.26$ N/m (Figure 7F). From our model, we found that a $\delta H$ of at least 1.2 nm was needed for $k = 0.26$ N/m $> k_c$ (Figure 9D). We note that $\delta H > 1.2$ nm can easily be attained with the 10 kDa PEG (i.e., $L_c = 60$ nm $> 1.2$ nm). Furthermore, the rupture peaks observed in Figure 8F are 2.3 nm apart, supporting our model prediction that $\delta H$ needs to be $> 1.2$ nm to observe multiple β-peptide rupture events using 10 kDa PEG tethers and AFM tip cantilevers with $k = 0.26$ N/m.

CONCLUSION

A key conclusion of our study is that the mechanical properties of short tethers (e.g., SSMCC) cause the pull-off forces measured with an AFM tip at β-peptide-functionalized surfaces to be dominated by a single AFM tip–oligopeptide binding interaction. This finding holds true even for experimental conditions for which multiple β-peptides lie within the contact area of the AFM tip and for which the probability of nonadhesive tip–sample interaction is low. In contrast, when long (>10 nm) tethers are used to immobilize the β-peptides, we measured adhesion forces to arise from the interactions of multiple β-peptides with the AFM tip. Our experimental findings are supported by a mechanical model that confirms that short SSMCC tethers do indeed facilitate the selection of single-molecule interactions in the presence of multiple binding interactions. In our model, the short tethers are extended to different degrees by the interaction of the peptides with the AFM tip (defined by $\delta H$ and eq 10). Because of the nonlinear mechanical properties of the short tethers, these distinct extensions cause the individual β-peptides to experience different loading forces and thus contribute unequally to the measured rupture force ($F_{rup,T}$). Overall, the use of short (<10 nm) tethers to measure single-molecule interactions on a surface presenting multiple binding groups is a strategy that appears to be potentially broadly useful as this approach may alleviate the need for (i) low binding probabilities, (ii) resolvable multiple bond rupture events, and (iii) AFM tip cantilevers with large spring constants.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b04618.

Discussions on determination of the 10 kDa PEG tether contour length, surface immobilization of the 10 kDa PEG tether, distribution of 10 kDa PEG tethered β-peptide rupture lengths, AFM imaging, and XPS (PDF)

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Notes

The authors declare no competing financial interest.

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