Dynamic-force spectroscopy measurement with precise force control using atomic-force microscopy probe

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The accuracy of dynamic-force spectroscopy (DFS), a promising technique of analyzing the energy landscape of noncovalent molecular bonds, was reconsidered in order to justify the use of an atomic-force microscopy (AFM) cantilever as a DFS force probe. The advantages and disadvantages caused, for example, by the force-probe hardness were clarified, revealing the pivotal role of the molecular linkage between the force probe and the molecular bonds. It was shown that the feedback control of the loading rate of tensile force enables us a precise DFS measurement using an AFM cantilever as the force probe. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2355432]

I. INTRODUCTION

The properties of noncovalent bonding between organic molecules have been extensively studied not only for understanding the biochemical reactions in living bodies but also for practical applications, realizing efficient biochemical materials with the ability of molecular identification or producing complex nanodevices by using self-assembly techniques. In this context, Evans and co-workers developed dynamic-force spectroscopy (DFS) in 1997,[1,2] which had distinct advantages over other thermodynamic experimental methods. DFS measurement provides not only an energy barrier height for the unbinding reaction but also a length scale to which the interaction between two molecules extends. Such a microscopic analysis of intermolecular interactions is of great importance in understanding the origin of the function of a molecule from its complex three-dimensional structure.

In DFS measurement, rupture force, the force required to rupture the molecular bond, is measured under a gradually increasing tensile force applied to a bonded molecular pair. To date, the biomembrane force probe (BFP) has been mainly used as the force probe in DFS measurements instead of the atomic-force microscopy (AFM) probe, although the latter is much more popular as an ultrasensitive force probe in other applications. This is because of the belief that a softer force probe is more accurate for DFS measurement. In this study, we reconsider this issue by revealing the role of the molecular linkage between the force probe and the molecular bonds. The advantages of using an AFM probe for DFS measurement are demonstrated.

II. DYNAMIC-FORCE SPECTROSCOPY

When a molecular pair is ruptured by a tensile force in a DFS measurement, the obtained rupture forces have a linear relationship to the logarithmic loading rate of the tensile force.[1–4] This is a consequence of the negligible rebinding rate of once-ruptured molecules, differing from the case of the unzipping model which has competitive unbinding and rebinding rates.[5–7] From the obtained linear relationship, we can obtain information about the *energy landscape* of the molecular interaction, i.e., energy barrier position x_b and natural lifetime of the molecular linkage t_{off}.

When the rupture force of a molecular pair is repeatedly measured, the rupture force is distributed over a finite range. The shape of its probability density function $P\{f_{\mathrm{rup}}\}$ depends on x_b, t_{off}, loading rate of tensile force r_0, and thermal energy k_BT as given by Evans.[4] We note that the equation given can be further transformed into

$$P\{f_{\mathrm{rup}}\} = C \exp\{(f_{\mathrm{rup}} - f^*)/f_\beta\}\exp[1 - \exp\{(f_{\mathrm{rup}} - f^*)/f_\beta\}], \tag{1}$$

with the normalizing constant C, the thermal energy of the system in the force dimension $f_\beta = k_BT/x_\beta$, the distance between the energy barrier and the most stable point measured along the reaction path $x_\beta = x_b \cos\theta$, and the most probable rupture force f^* that gives the maximum value of $P\{f_{\mathrm{rup}}\}$, where

$$f^* = f_\beta \ln(t_{\mathrm{off}} r_0 / f_\beta) = f_\beta\{\ln(r_0) - \ln(f_\beta / t_{\mathrm{off}})\}. \tag{2}$$

As is obvious from Eqs. (1) and (2), the peak width of the density function is determined only by x_β and k_BT, whereas the peak position also depends on t_{off} and r_0. Thus, when a molecular system is measured with different loading rates, the distribution of the rupture force is only shifted along the force axis without changing its peak shape. Consequently, although previous DFS measurement was only concerned with the dependence of the peak position on the loading rate of the tensile force, if an ideal experiment could be performed, the peak width and peak position of the rupture force distribution for a single loading rate would provide us with both x_β and t_{off}.

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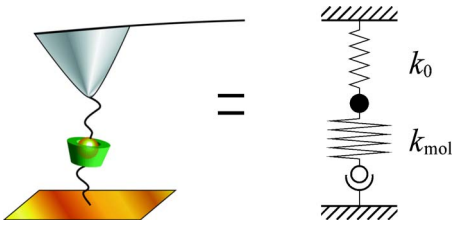

FIG. 1. (Color online) Soft molecular linkage acting as *molecular rubber*.

III. ACCURACY OF DFS MEASUREMENT

To date, it is believed that a soft force probe is better than a hard force probe for DFS measurement. As a result, the BFP has mainly been adopted for the force probe, which is a few orders of magnitude softer than the most commonly used ultrasensitive force probe, AFM cantilevers. Here, we review the issues that affect the accuracy of DFS measurement and reconsider why the harder force probe has not been preferred. We demonstrate that understanding the hidden role of the flexible molecular chain, which connects the force probe and the molecular pair, fundamentally changes the concept of DFS measurement that has been accepted until now.

A. Molecular linkages

In DFS measurement, a bonded molecular pair is fixed onto the apex of a force probe and a substrate surface via molecular chains of ~30 nm, as shown in Fig. 1. Such molecular chains act kinetically as molecular rubber, whose force-distortion curves can be described by freely jointed chain (FJC) model or wormlike chain (WLC) model[7,8] and are highly nonlinear, particularly for small forces. The nonlinearity of the force-distortion curves often makes it difficult to control the loading rate of tensile force, as discussed below, particularly when the molecular pair is more flexible than the force probe. However, a flexible molecular chain, i.e., a long molecular chain, is favorable from other aspects. The flexibility of a molecular chain provides the molecular pair with freedom of motion, which enables the rupture process to occur along the most energetically favorable reaction path. In addition, long molecular linkages separate the apex of the force probe and the substrate, reducing the effect of the long-range interaction between the probe and the substrate.

In general, the force probe has a much larger mass than the molecular system and has large damping in a liquid environment. Thus, its motion is many orders of magnitude slower than that of the molecular system, whose trial frequency is usually $>10^9$ Hz. In such a situation, the molecular chain, rather than the force probe, effectively exerts tensile force on the molecular pair. However, the force probe merely measures the average force that the molecular chain applies to the molecular pair. One must keep this point in mind when discussing the accuracy of the measurement.

B. Thermal fluctuation of force probe

In the situation shown in Fig. 1, the thermal fluctuation amplitude of the force-probe deformation can be expressed as $\Delta x_{\mathrm{fp}}=\sqrt{k_BT/(k_{\mathrm{fp}}+k_{\mathrm{mol}})}$, with the spring constant of the force probe k_{fp} and the stiffness of the molecular linkage k_{mol}. In the force measurement, this fluctuation corresponds to the uncertainty of the measured force value $\Delta f_{\mathrm{fp}}=k_{\mathrm{fp}}\Delta x_{\mathrm{rms}}=\sqrt{k_{\mathrm{fp}}k_BT/(1+k_{\mathrm{mol}}/k_{\mathrm{fp}})}$. This equation suggests that a softer force probe, namely, one with low k_{fp}, gives a smaller uncertainty; thus, this indicates an advantage of using a softer probe. However, this is true only if the measurement bandwidth covers the whole spectral distribution of the thermal fluctuation. When the force probe has a high resonant frequency and a high quality factor, the frequency distribution of its thermal fluctuation is almost fully out of the measurement bandwidth. In such cases, a very accurate measurement can be realized.[9] Therefore, since a harder force probe gives a higher resonant frequency and a higher quality factor in a liquid environment, its thermal noise level can be as low as that of softer probes.

One might be afraid that accurate measurement of tensile force with a limited bandwidth is not sufficient but precise control of the tensile force along with the loading rate is required. If the tensile force contains a large fluctuation, an instantaneous large force can break the molecular bond even if the average force is small. Thus, the fluctuation of tensile force must be suppressed, regardless of the measurement bandwidth. Then, does a hard probe cause larger fluctuation on the molecular bond?

The answer is "no." According to statistical mechanics, the thermal fluctuation that the molecular pair receives is always $k_BT/2$. Namely, the amplitude of thermal fluctuation is independent of the hardness of the force probe. For better understanding, imagine that the force probe is thermally fluctuating around an averaged position and momentarily bent towards the sample by the fluctuation. Then, the rupture probability becomes smaller because the length of the molecular chain is decreased, although the apparent tensile force is increased by the bending. The deformation of the force probe is only induced by thermal fluctuation and the molecular pair does not experience the exact force of the instantaneously measured value. Consequently, one can use hard force probes in DFS measurements, as long as the measurement precision of tensile force is sufficiently high within a required bandwidth.

C. Rebinding probability

Rebinding of the ruptured molecules was investigated by Evans as related to the disadvantage of using a hard force probe.[4] Since hard probes do not deform much for a small rupture force, the two molecules are not pulled far apart after the rupture. For example, a force probe with a 100 pN/nm spring constant pulls the molecules apart only by 1 Å when the rupture force is 10 pN. In this case, these two molecules may have a high probability of rebinding, which causes a critical discrepancy from the ideal DFS theory. However, in a real system, the coupled stiffness of the force probe and molecular chains is given by $k_{\mathrm{fp}}k_{\mathrm{mol}}/(k_{\mathrm{fp}}+k_{\mathrm{mol}})$. Thus, even when the force probe is very hard, the contraction of the soft molecular chain separates the two molecules if the molecular chain is sufficiently flexible. Moreover, the motion of the two

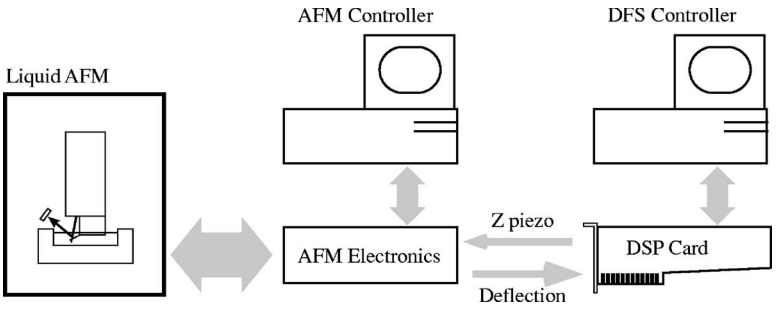

FIG. 2. DFS measurement system.

molecules has three-dimensional freedom after the rupture. Therefore, the rebinding probability is negligible when we use sufficiently long molecular chains.

D. Constant loading rate

When a force probe ideally follows Hooke's law, the retraction of the base of the force probe at a constant velocity gives a constant loading rate of the tensile force in rupture force measurement. However, the actual force probe is combined with a molecular chain that exhibits a nonlinear force-distance relationship. As a result, when the stiffness of the force probe is greater than that of the molecular chain, retraction of the base at a constant velocity does not give a constant loading rate. This problem was previously pointed out and has been a difficulty in DFS measurement.[10]

As will be discussed, if the measurement bandwidth of the force probe is sufficiently wide, we can solve this problem by performing feedback control of the retraction velocity to maintain a constant loading rate.

E. Stability of measurement system

The instability of the measurement system due to thermal drift and the creep/hysteresis of the piezoelectric device also affect the measurement accuracy. Such instability causes a change in the probe-substrate distance. When the loading rate is determined by the constant velocity of the force probe, this instability affects the DFS measurement. Since this effect is proportional to the stiffness of the force probe, a softer probe seems to be superior. However, the effective stiffness of the force probe is determined by the combination of the probe stiffness and that of the molecular linkage. Thus, if the molecular chain is sufficiently flexible, the high stability of the measurement system can be maintained. Moreover, if one uses the feedback control of the loading rate proposed above, this type of instability is automatically compensated.

The instability of the detection of probe deformation should be independently discussed. The response of a hard force probe is affected more by such instability than that of a soft force probe. Thus, the detection signal often drifts gradually during a long-term measurement. This difficulty should be solved by calibrating the natural position of the force probe every time before starting each rupture force measurement, particularly when a hard force probe is used.

F. Advantage of using AFM probe in DFS measurement

Since its invention two decades ago, the measurement accuracy of AFM has undergone many improvements. Now, many good AFM systems are commercially available. Such systems generally have a higher resolution and a wider bandwidth for the detection of probe deformation than those of BFPs and laser optical tweezers (LOTs).[11–13] Therefore, AFM systems, with a relatively hard probe, possess a high accuracy of force detection. In addition, the faster response of the measurement system enables feedback control of the loading rate of tensile force, as demonstrated in the next section.

IV. EXPERIMENT

A. DFS measurement system

The block diagram of our measurement system is shown in Fig. 2. An AFM system with a liquid cell (Molecular Imaging, Pico SPM) is connected to a commercial AFM electronics system (Digital Instruments Nanoscope III) that is modified to extract the cantilever deflection signal and to mix additional signals into *Z*-piezoelectric voltage. After the cantilever is placed to gently touch the substrate, the feedback circuit of the AFM controller is turned off. Then, the DFS measurement is fully controlled by a digital signal processor (DSP) board (*Toro*, Innovative Integration) with a 150 MHz TMS320C6711 (Texas Instruments) installed into a PCI slot of another personal computer.

The algorithm of the DFS force curve measurement is as follows. (1) The cantilever is retracted 150–200 nm from the substrate, and the deflection signal is recorded to calibrate the natural position of the cantilever with zero probe-sample interaction. (2) The cantilever is moved towards the substrate at a constant velocity, and when the deflection signal detects 100–200 pN of a specified repulsive force from the surface, the feedback control of *Z*-piezoelectric displacement is turned on to maintain the deflection signal at the same value. (3) The cantilever and the substrate are kept gently touching for 10 ms–1 s to form a molecular bond between them. (4) The reference value for *Z*-piezoelectric feedback is changed in accordance with the desired loading rate. As a result, the cantilever is retracted from the substrate at a constant loading rate until bond rupture. During this process, the deflection signal and *Z*-piezoelectric displacement are recorded at a 100 kHz sampling rate onto a hard disk drive. (5) Once the cantilever is 150–200 nm from the substrate, the control jumps back to step (1) and the next force curve measurement is started.

With this procedure, we can calibrate the natural position of the force probe every time before starting the force curve measurement, to maintain an accurate contact pressure and contact time of the probe apex with the substrate. In addition, since *Z*-piezoelectric feedback automatically increases retracting velocity after bond rupture, this procedure can decrease the total acquisition time, which is efficient particularly for small loading rates.

B. Preparation of molecules on probe apex and substrate

A gold-coated cantilever (*Bio-Lever*, Olympus, 30 pN/nm, rectangular shape) was immersed into a solution of 8-amino-1-octanethiol, hydrochloride (Sigma-Aldrich, 1 mM in ethanol) for 48 h to form a close-packed self-assembled monolayer (SAM) with an amino group at the surface. After rinsing with ethanol, the cantilever was immersed into a solution of biotin-PEG3400-COO-NHS [Shearwater Polymers, 0.1 mM in ethanol:DMF (dimethylformamide)=99:1] for 20 h to fix biotin onto the probe apex. Finally, the cantilever was rinsed with ethanol.

A gold thin film (100 nm) was evaporated onto a freshly cleaved mica surface in high vacuum at 400 °C and was flame annealed using a hydrogen gas burner for ~30 s. Then, the gold substrate was immersed into a solution of 1,8-octanedithiol (Sigma-Aldrich, 1 mM in ethanol) for 48 h to form a close-packed SAM with a thiol group at the surface. After rinsing with ethanol, the substrate was immersed into a solution of streptavidin-maleimide (Sigma-Aldrich, 10 mg/l in phosphate buffered saline (PBS), *p*H 7.4:DMF =99:1) for 5 h to fix streptavidin molecules to the substrate. Finally the substrate was rinsed with PBS.

The formation of closely packed SAMs on the probe apex and the substrate was necessary to decrease the strong interaction between the gold layers on both surfaces. DFS measurements were performed at room temperature in PBS, in which a small amount of free biotin molecules was dissolved. The free biotin molecules occupy the reaction site of the streptavidin molecules on the substrate surface and reduce the probability of biotin-streptavidin bonding down to 10% for each measurement. This procedure is necessary to measure the rupture force at the single-molecule level, by avoiding the formation of multiple molecular bonds.

V. RESULTS AND DISCUSSION

A. Rupture force measurement with feedback control

A typical force curve of streptavidin-biotin obtained using our system is shown in Fig. 3. The force signal during retraction is plotted against (a) time and (b) cantilever displacement. At first, in region A, the probe apex and the substrate are in contact. Then, in region B, tensile force is applied to the molecular pair through the molecular linkage [polyethylene glycol (PEG)]. At the end of region B, the molecular pair is ruptured, and the cantilever returns to its natural position. Although the force-distance curve is nonlinear in region B, as shown in Fig. 3(b), the loading rate is kept almost constant by the feedback circuit, as shown in Fig. 3(a). The low-pass signal with a cutoff frequency of 200 Hz

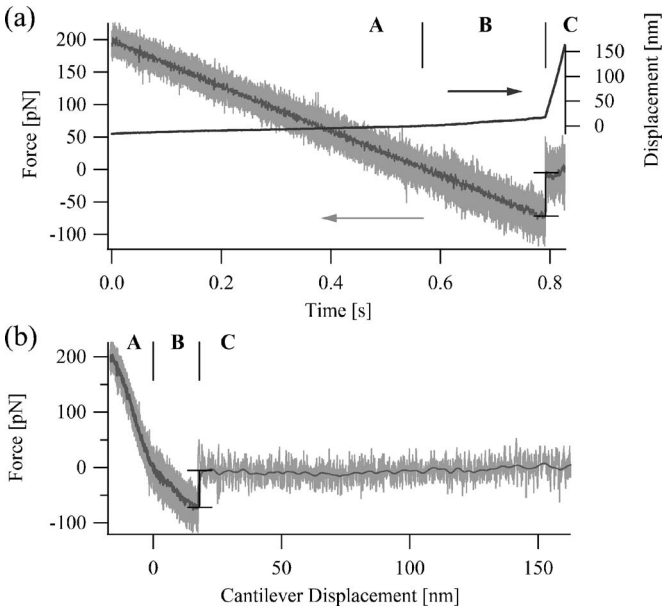

FIG. 3. Typical force curve of rupture force measurement. (a) Force and cantilever displacement plotted against time. (b) Force plotted against cantilever displacement. The gray and black curves in the force plot are raw and low-pass filtered signals with 50 kHz and 200 Hz bandwidths, respectively.

(dark gray) contains a thermal fluctuation of approximately 3 pN, whereas that of the raw data sampled at 100 kHz (gray) is approximately 20 pN.

B. Histogram of rupture force

Thousands of force curve measurements were performed using the method described above for each loading rate and approximately 10% of them exhibited a rupture, as expected. The rupture forces are summarized in histograms, as shown in Fig. 4. The extraction of the rupture force is performed by fitting the corresponding part of the force curves with a tilted step function. With this fitting procedure, an accuracy of ~1 pN was achieved for loading rates below 10^5 pN/s. For larger loading rates, reduced number of data points available for fitting and insufficient feedback control of rupture force decreased the measurement accuracy.

From the fitting of the histograms using Eq. (1), we obtain the values of f^*, x_β, and t_{off} for each loading rate. As can be seen, for example, in Fig. 4(c), whereas some of the plots show good agreement with the theoretical expectation, others do not. More specifically, the plots tend to have a small but nonzero probability in high-rupture-force areas and less probability near zero force. The former may be due to the small probability that multiple molecular bonds are formed between the tip and the substrate, whereas the latter is due to the limitation of the force-signal detection of distinguishing a very small rupture force in the thermal noise. These two factors result in the peak positions f^* of the fitting parameter being higher than the maximum peak positions of the histograms (indicated by the black arrowhead in the plots).

C. Rupture force dependence on loading rate

The most probable rupture forces f^* obtained by the curve fitting of the histograms using Eq. (1) (filled circles)

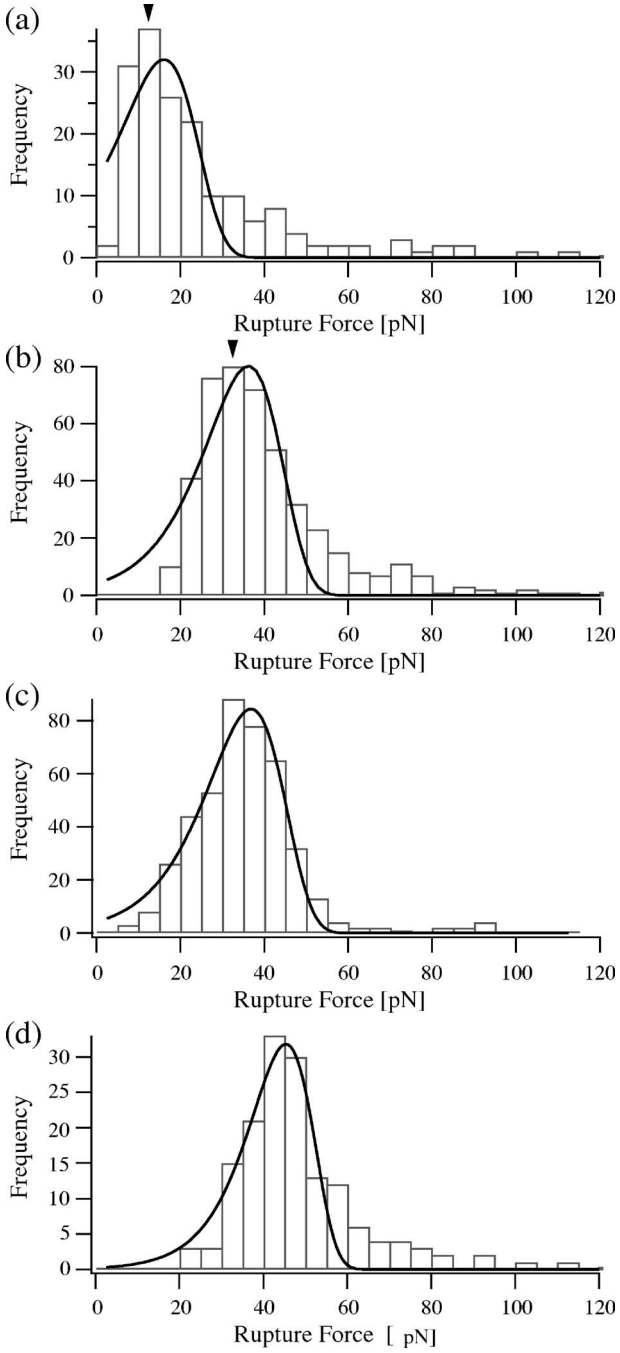

FIG. 4. Histograms of rupture force for loading rates of (a) 34.7 pN/s, (b) 967 pN/s, (c) 3320 pN/s, and (d) 16 300 pN/s. The black lines are the fitting functions given by Eq. (1).

are plotted against the logarithm of loading rate $\ln(r_0)$, as shown in Fig. 5. There is a clear linear relationship of $f^* = (4.67\ \text{pN})\{\ln(r_0) - \ln(6.04\ \text{pN/s})\}$. From the comparison of the gradient and x intersection of the line with Eq. (2), we can obtain the parameters $x_\beta = 0.89$ nm and $t_{\text{off}} = 1.3$ s.

The x_β values obtained by fitting each rupture force histogram using Eq. (1) are also shown at each data point. Since any experimental errors result in the peak widths of the rupture force distribution becoming wider, these x_β values are smaller than that obtained from the curve slope. However, the consistency of the values obtained by the two different methods indicates the validity of our measurement.

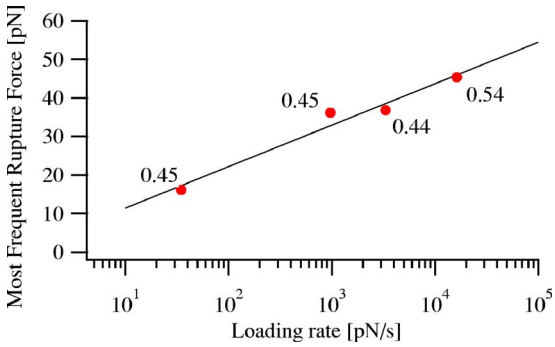

FIG. 5. (Color online) Loading rate dependence of rupture force. The numbers next to the data points represent the barrier positions x_β obtained by the width analysis of each histogram and the black line shows the linear fitting.

The deviation of the data points from the linear relationship is approximately the same order of magnitude as that of the previous experiment on the same system measured using BFP, which has a spring constant more than one order of magnitude softer (0.1–3 pN/nm) than our AFM cantilever.[2] This result demonstrates the validity of using a hard cantilever for DFS measurement.

In comparison with the previous experiment by Merkel *et al.*,[2] x_β obtained from the slope of the curves in Fig. 5 is almost consistent with their result, whereas t_{off} obtained here is much smaller. Although the discrepancy in the values of t_{off} is not yet fully understood, the preliminary results of our ongoing experiments suggest that the different chemical attachment states of the streptavidin molecules onto the substrates affect the result.

VI. CONCLUSION

In order to show why and how one can utilize widely available AFM cantilever for precise DFS measurement, previously suggested disadvantages of using hard force probes in DFS measurement were reconsidered. With understanding the pivotal role of the molecular chain between the force probe and the molecular complex, following consequences are derived. Use of a hard force probe does not increase measurement uncertainty due to the thermal fluctuation nor the rebinding rate of a ruptured molecular pair. A feedback control of tensile force in rupture force measurement can solve the difficulty in maintaining a constant loading rate and compensate the instability of the measurement system. As a result, the same-order accuracy as that by DFS measurement using a biomembrane force probe was obtained by an AFM-based measurement system. These results pave the way for DFS measurement to become more widely available.

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