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Direct measurement of single-molecule visco-elasticity in atomic force microscope force-extension experiments

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Abstract Measuring the visco-elastic properties of biological macromolecules constitutes an important step towards the understanding of dynamic biological processes, such as cell adhesion, muscle function, or plant cell wall stability. Force spectroscopy techniques based on the atomic force microscope (AFM) are increasingly used to study the complex visco-elastic response of (bio-)molecules on a single-molecule level. These experiments either require that the AFM cantilever is actively oscillated or that the molecule is clamped at constant force to monitor thermal cantilever motion. Here we demonstrate that the visco-elasticity of single bio-molecules can readily be extracted from the Brownian cantilever motion during conventional force-extension measurements. It is shown that the characteristics of the cantilever determine the signal-to-noise (S/N) ratio and time resolution. Using a small cantilever, the visco-elastic properties of single dextran molecules were resolved with a time resolution of 8.3 ms. The presented approach can be directly applied to probe the dynamic response of complex bio-molecular systems or proteins in force-extension experiments.

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Abbrevations AFM: Atomic force microscope · PSD: Power spectral density · S/N: Signal-to-noise · l: Length · t: Thickness · w: Width · ν_{res}: Resonance frequency

Introduction

The mechanical properties of biological macromolecules play an important role in fundamental biological processes, including cell adhesion, muscle function, and gene transcription (Bustamante et al. 2004; Howard 2001). Therefore bio-molecular mechanics have been extensively studied in single-molecule force-extension experiments using the atomic force microscope (AFM) (Binnig et al. 1986) or optical and magnetic tweezers (Leckband and Israelachvili 2001). In AFM force spectroscopy measurements, a single molecule is tethered between the tip of the AFM cantilever and a sample surface. Then the tip-surface separation is continuously increased and, as a consequence, the molecule is gradually extended thereby revealing its elastic response in terms of the force-extension relationship (Rief et al. 1997a, b). Importantly, most bio-molecular interactions exhibit elastic and viscous forces, which should be analyzed independently to allow a deeper understanding of their contribution to dynamic biological processes. Extended AFM force spectroscopy experiments have been developed to measure such visco-elastic responses of a wide class of (bio-)molecules, including nucleic acids (Liu et al. 1999), receptor–ligand complexes (Chtchelgl-ova et al. 2004), proteins (Forbes and Wang 2004; Janovjak et al. 2005), polysaccharides (Humphris et al. 2000, 2002) and synthetic polymers (Kienberger et al. 2000). In these force modulation spectroscopy experiments, the AFM cantilever is sinusoidally oscillated while a single molecule is extended between the tip and the surface. Monitoring the amplitude and phase response of the cantilever then allows conservative (elastic) and dissipative (viscous) interactions to be distinguished.

It was shown that the stretching of unfolded proteins (Forbes and Wang 2004; Janovjak et al. 2005), nucleic acids (Liu et al. 1999), and poly(ethyleneglycol) (Kienberger et al. 2000) is dominated by purely elastic interactions. In apparent contrast, the unfolding of secondary structure elements of the membrane protein bacteriorhodopsin and the giant muscle protein titin as well as the chair-boat transitions in the polysaccharide dextran could be correlated to dissipative interactions (Forbes and Wang 2004; Humphris et al. 2000, 2002; Janovjak et al. 2005).

Kawakami and co-workers recently introduced a different approach to measure the visco-elastic properties of single molecules (Kawakami et al. 2004). Instead of using a continuous extension protocol, single dextran molecules were clamped at pre-defined forces using a feedback loop. Then the thermal motion of the AFM cantilever was analyzed for a few seconds to obtain the molecular visco-elasticity as a function of the applied tension. Here, we show that single-molecule visco-elasticity can be directly extracted from the thermal cantilever motion during conventional force-extension measurements, i.e. as a function of tip-sample separation. Dextran was chosen as a model system for our study since its visco-elastic properties have already been extensively studied (Humphris et al. 2000, 2002; Kawakami et al. 2004, 2005).

Experimental

Sample preparation

Dextran (average molecular weight 2 MDa) was purchased from Sigma. 200 µl of a 5% w/w dextran solution in nanopure water was allowed to dry in air on glass coverslips at 37°C. Samples were then rinsed for 1 min under a flow of nanopure water. Experiments were performed in 150 mM KCl, 10 mM Tris–HCl, pH 7.8 at 160 nm/s pulling speed.

AFM instrumentation

A commercial AFM (PicoForce, di-Veeco, Santa Barbara, CA, USA) was extended with a PC equipped with 16-bit data acquisition electronics (6052E, National Instruments, Munich, Germany). The cantilever deflection signal was first low-pass filtered at 100 kHz with a passive hardware filter to avoid aliasing and then digitized at 300 kHz using Igor Pro (Wavemetrics, Lake Oswego, OR, USA). The spring constants, k, and resonance frequencies, ν_{res}, of the cantilevers were calibrated in buffer using thermal fluctuation analysis (Butt and Jaschke 1995; Florin et al. 1995). The cantilevers used were the short thing-legged NP-S ($k \approx 0.31$ N/m, $\nu_{res} \approx 10$ kHz, length (l)≈ 115 µm, width (w)≈ 25 µm, thickness (t)≈ 0.4 µm) and Microlever B (MLCT-B, $k \approx 0.04$ N/m, $\nu_{res} \approx 3.5$ kHz, $l \approx 200$ µm, $w \approx 20$ µm, $t \approx 0.6$ µm) from di-Veeco and the BioLever-A (BL-A, $k \approx 0.05$ N/m, $\nu_{res} \approx 10$ kHz, $l \approx 60$ µm, $w \approx 30$ µm, $t \approx 0.18$ µm) and "Mini BioLever" BL-AC40TS (M-BL, $k = 0.117$–0.131 N/m, $\nu_{res} \approx 30$ kHz, $l \approx 38$ µm, $w \approx 16$ µm, $t \approx 0.2$ µm; (Toda et al. 2004)) from Olympus (Tokyo, Japan).

Extracting visco-elastic properties

Deflection curves were analyzed using custom macros and built-in features of Igor Pro. The curves were first high-pass filtered at 400 Hz (also to reduce contributions of $1/\nu$ noise) and then divided into windows. Each window was processed by calculating the power spectral density (PSD) with 512-point hanning-type functions overlapping by 50%. Thus nine Fourier spectra were averaged for each 8.3 ms raw data window (Figs. 1, 3) and the average PSD were fitted with (2). Calculation and fitting of the PSD was independently double-checked using the thermal tune function of the AFM and Origin software (Northampton, MA). To obtain the molecular visco-elastic response according to (3, 4, 5), the visco-elasticity curves recorded during surface approach were smoothed and subtracted from the corresponding visco-elasticity curves recorded during surface retract as recently described (Janovjak et al. 2005).

Results and discussion

We present a simple approach to measure the visco-elastic properties of single (bio-)molecules based on the Brownian motion of an AFM cantilever. In contrast to the force-clamp technique recently introduced by Kawakami (2004), the thermal cantilever motion was analyzed as a function of tip-sample separation while a single molecule was continuously stretched between the tip of the cantilever and the sample surface. The polysaccharide dextran was chosen as a model system since its force-extension behavior and visco-elastic properties had already been extensively studied (Humphris et al. 2000, 2002; Kawakami et al. 2004, 2005). The force curve shown in Fig. 1a was recorded while stretching a single dextran molecule and thus exhibited the characteristic force-extension pattern (Marszalek et al. 1998; Rief et al. 1997b), which is only briefly discussed here. At small extensions (here < 70 nm), almost all pyranose rings are in the chair conformation and an increase in force is measured due to entropic stretching of the polysaccharide chain. As soon as the applied force reaches ≈ 0.9 nN, the pyranose rings undergo the chair-boat transition, which elongates the dextran chain by about 18% and appears as a plateau-like region in the force curve (Marszalek et al. 1998; Rief et al. 1997b). After most of the rings have assumed the boat conformation, an increase in force is detected prior to the detachment of the molecule from the tip.

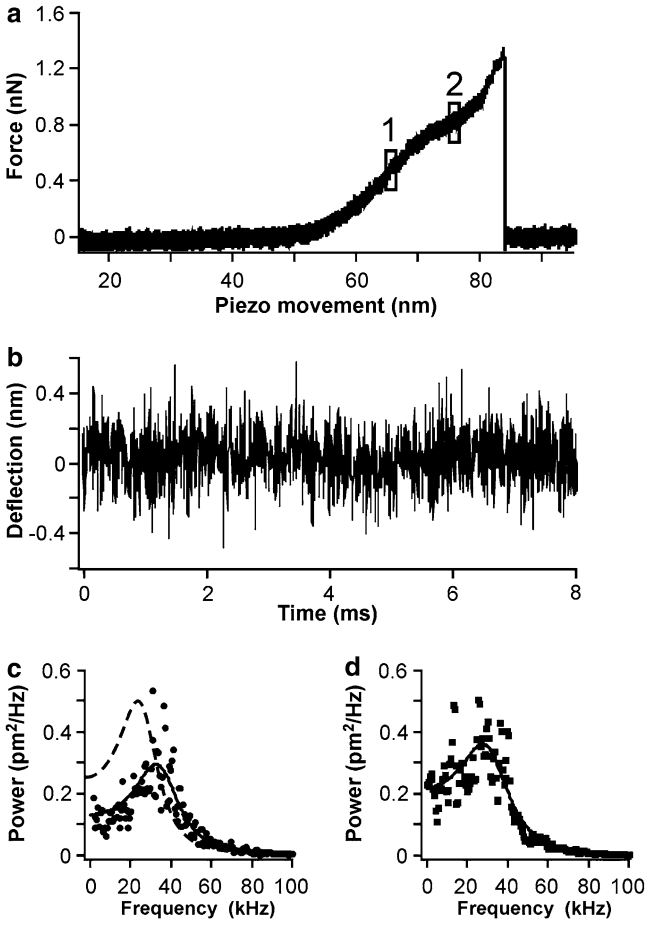

Fig. 1 Analysis of thermal cantilever motion in a force-extension experiment. **a** Typical force curve of a single dextran molecule recorded with a small cantilever. The most characteristic feature is the plateau-like region at a force of ≈0.9 nN. As examples, the analysis of two segments (marked 1 and 2) is shown in (**b**–**d**). **b** Deflection signal in segment 1 shown as a time series after hi-pass filtering. **c**, **d** PSDs calculated from the deflection of the free cantilever 160 nm form the surface (*dashed line* in **c**), the deflection in segment 1 (*markers* in **c**) and segment 2 (**d**). The PSDs are well described by fitting with (2) (*solid lines*) and reveal the visco-elastic properties of the system at that specific instance

The first step in our analysis was to extract the thermal cantilever motion from the force curve using high-pass filtering. Then the curve was divided into small windows (Fig. 1b) and for each window the thermal noise PSD was calculated (Fig. 1c, d). According to the simple harmonic oscillator model (Sader 1998; Sarid 1994), the motion of a thermally excited AFM cantilever can be described by the following differential equation

$$Ex + \gamma\dot{x} + m\ddot{x} = f(t) \tag{1}$$

Here, $f(t)$ is the stochastic thermal force, x denotes the tip displacement, E is the elasticity, γ the damping coefficient and m the effective mass of the cantilever-molecule system. This simple model then allows E, γ, and m to be obtained from the PSD according to (Roters and Johannsmann 1996)

$$\left\langle \left| x(v)^2 \right| \right\rangle = \frac{2k_B T\gamma}{\left[E - m(2\pi v)^2\right]^2 + \gamma^2(2\pi v)^2} \tag{2}$$

where $\left\langle \left| x(v)^2 \right| \right\rangle$ is the frequency v dependent PSD. Since the cantilever and the molecule act in parallel (Pethica and Oliver 1987), the visco-elastic properties of the system can be expressed by

$$E = E_c + E_{\text{mol}}, \tag{3}$$

$$\gamma = \gamma_c + \gamma_{\text{int}}, \tag{4}$$

$$m = m_c + m_{\text{mol}} \tag{5}$$

where the index c denotes the quantities associated with the unperturbed lever. The molecule contributes linearly (E_{mol}) to the system's elastic modulus E, and, with its effective mass m_{mol}, to the total mass m. The damping coefficient is decomposed into the initial contribution of the cantilever γ_c, and the contribution γ_{int} arising from the interaction. Conceptually, γ_{int} contains the dissipation by the molecule itself as well as the damping induced by the altered boundary condition at the tip (force clamped end) (Rabe et al. 1996; Stark et al. 2004; Wu et al. 2005). It was shown that E_c, γ_c, and m_c are readily extracted from the motion of a free cantilever and allow hydrodynamic and surface effects to be excluded (Janovjak et al. 2005; Roters and Johannsmann 1996). Thus, by applying (2, 3, 4, 5), E_{mol}, γ_{int}, and m_{mol} are obtained. In the following, we will focus our discussion on the visco-elastic response of the molecule (elastic modulus and damping) since a change in effective mass is usually not detected in single-molecule manipulation experiments (Kawakami et al. 2004, 2005; Pethica and Oliver 1987).

To evaluate the effect of the window size on the signal-to-noise (S/N) ratio of our experiments, we analyzed force-extension curves where no molecule had attached to the AFM tip. For these curves, the peak noise in the elasticity and damping coefficient was measured along the tip-sample separation in a range between 0 and 160 nm. One should note that there is only a negligible dependence of the elasticity and damping coefficient on tip-sample separation in this range (Fig. S1). As expected, this analysis showed that the peak noise in the elasticity and damping coefficient critically depended on the size of the window (Fig. 2). Increasing the window size increases the time period available to calculate the frequency components of the motion of the cantilever and thus results in smaller noise (Fig. 2a, b) and consequently a better apparent S/N ratio (Figs. 2c, d). We also noticed that the S/N ratio critically depended on the type of AFM cantilever used. In Fig. 2, the results for two types of cantilevers are shown. These cantilevers differed in their size, spring constants, and resonance frequencies (Experimental section). Choosing a novel small cantilever with high resonance frequency (≈30 kHz in buffer) significantly improved the S/N ratio of our dynamic measurements.

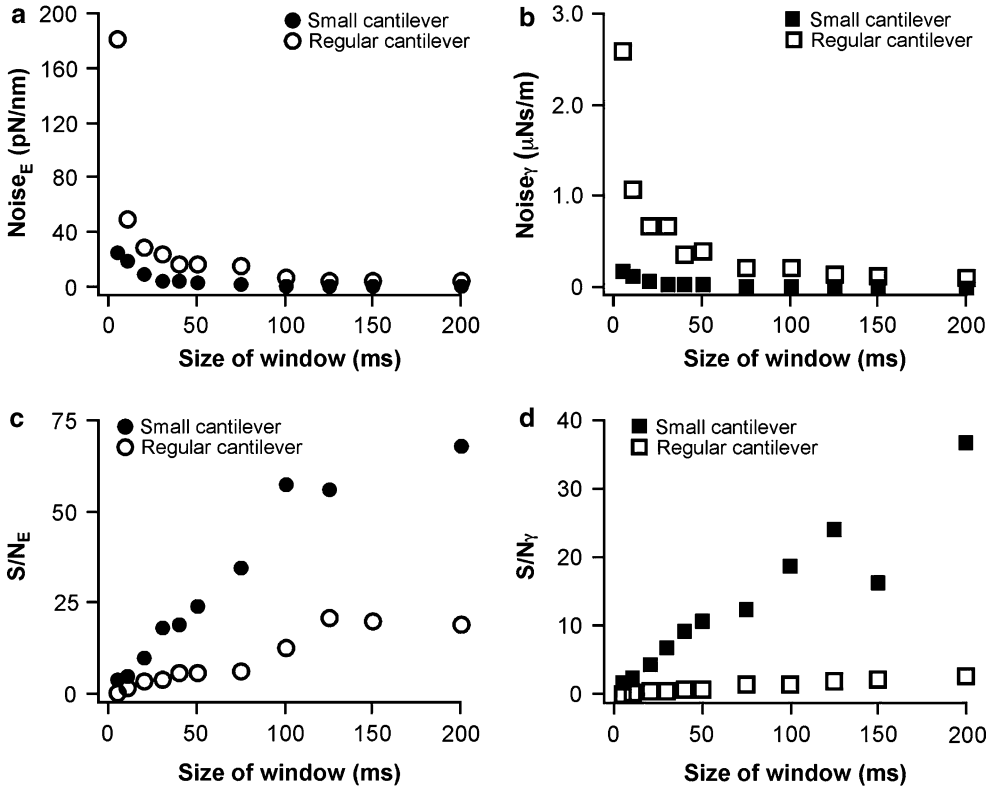

Fig. 2 S/N-ratio depends on windows size and cantilever. The noise in elasticity (**a**) and damping (**b**) measurements is shown as a function of the size of the window. As expected, increasing the time period available for monitoring the cantilever motion and calculating the PSD reduces the noise (**c**, **d**). The apparent S/N ratios were calculated by comparing a peak elasticity and damping of $E_{mol} = 100$ pN/nm and $\gamma_{int} = 0.3$ μNs/m with the noise values from (**a**, **b**). While both cantilevers showed similar performance in elasticity measurements (**c**) the damping information could only be extracted from the small cantilever measurements (**d**)

While both cantilevers had similar performance in measuring the elasticity (Fig. 2a, c), only the smaller cantilever offered a sufficient S/N ratio to resolve the damping coefficients (Fig. 2b, d). One probable reason for the better S/N ratio of the small cantilever is its significantly higher resonance frequency. However, other size-dependent effects must be responsible for the drastic difference in the S/N ratio when determining the damping coefficients. Thus we have chosen a fixed window size and measured the sensitivity of four different types of cantilevers, which span a wide range of sizes and resonance frequencies. In Fig. S2, their sensitivities are plotted as a function of their damping coefficients and resonance frequencies. These data highlight that the damping coefficients and thus the size of the cantilevers have a very strong influence on their dynamic S/N ratio.

For the polysaccharide dextran, a reasonable trade-off between S/N ratio and time-resolution was achieved using the small cantilevers and window size of 8.3 ms. At a pulling speed of 160 nm/s per second, this window size yields one dynamic measurement every ≈1.3 nm. If the pulling speed is reduced tenfold (to 16 nm/s) as in a recent paper by Kawakami et al. (Kawakami et al. 2005), one could obtain one measurement per 1.3 nm with an improved S/N ratio by a factor of ≈5.

Figure 3 shows the elasticity and damping coefficients associated with two dextran molecules. To allow a direct comparison with the results obtained using other approaches, we have plotted the molecular elasticity and damping coefficients as a function of the applied force (Fig. 3a, c). We also normalized the curves to show the monomeric elasticity (E_{mon}) and damping coefficient (γ_{mon}) as recently described (Kawakami et al. 2005). This is necessary as the extended dextran fragments typically have different lengths. The molecular response revealed the typical apparent visco-elastic behavior of single dextran molecules associated with the chair–boat-transition. Already at an applied force of ≈0.3 nN, an increase in E_{mon} and γ_{mon} was observed due to the stretching of the polysaccharide chain. A broad maximum was reached at a force of ≈0.6 nN before a decrease in molecular elasticity and damping coefficient is detected during the chair-boat transition. As for the low-force regime, further stretching of the polysaccharide chain again leads to increasing E_{mon} and γ_{mon}. As already mentioned above the apparent visco-elastic properties of single dextran molecules measured here are in good agreement to those recently reported (Kawakami et al. 2004, 2005) and possible physical origins were suggested there.

Here, we have also calculated the (molecular) relaxation times, τ, which are given by the ratio of interaction induced damping and elasticity following $\tau = \gamma_{int}/E_{mol}$ (Fig. S3). Surprisingly there is no clear dependence of

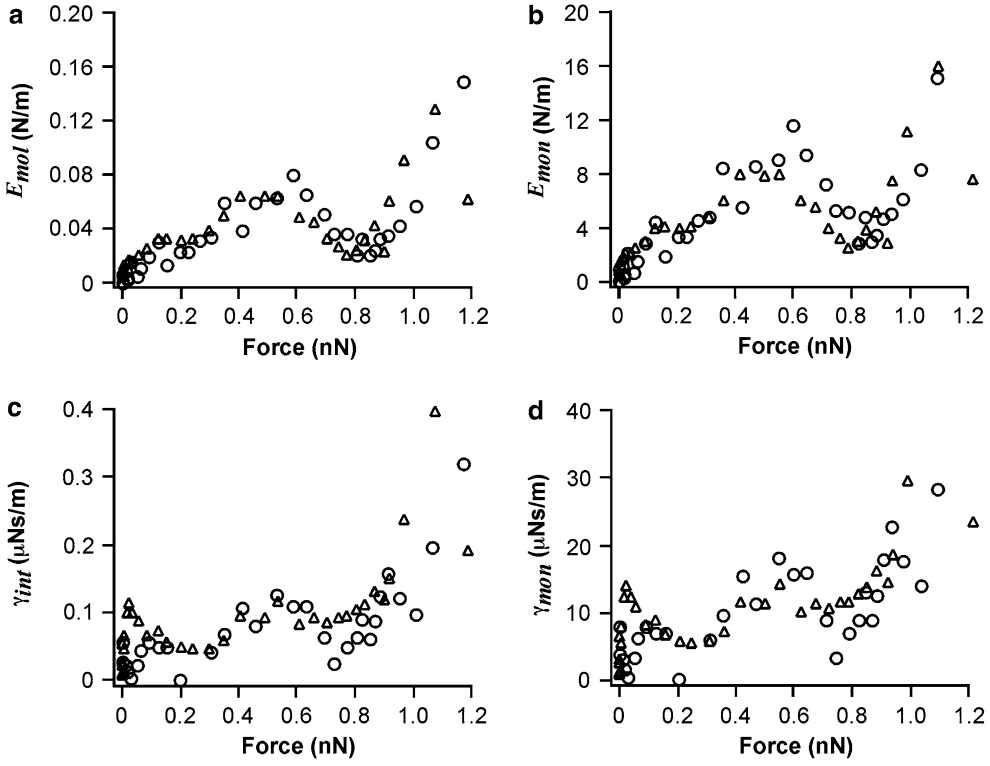

Fig. 3 Dynamic response of single dextran molecules. The molecular elasticity (**a**) and damping coefficient (**c**) of two single dextran molecules are shown as a function of the applied force. The two molecules stretched had lengths corresponding to 145 (*circles*) and 124 (*triangles*) monomers and show the typical visco-elastic behavior of dextran. Broad maxima in the elasticity and damping are detected at a force of ≈0.6 nN prior to a decrease in elasticity and damping during to the chair-boat-transition. **b, d** The curves from (**a**) and (**c**) were multiplied with the number of monomers to yield the response of an individual pyranose subunit

the relaxation time on the applied force and particularly there is no maximum near ≈0.6 nN. In addition, the relaxation times found here for dextran are much smaller than those recently reported for individual membrane proteins (Janovjak et al. 2005).

Importantly, we also noticed that the molecular damping coefficients seem to correlate with the molecular elasticities for large parts of the measurements (Figs. 3, S3). As mentioned above, such coupling of elastic and viscous interactions may indicate direct crosstalk of the elastic component into the apparent damping behavior (Stark et al. 2004). We are currently further investigating the influence of the cantilever dynamics in dynamic pulling experiments, also in the light of the increasing number of publications in this emerging field.

Concluding remarks

The past decade has seen a dramatic increase in our understanding of the mechanical properties of biological macro-molecules and their essential role in biological processes (Bustamante et al. 2004; Howard 2001). In single-molecule force-extension experiments, researchers have been studying the mechanical properties of a wide class of bio-molecules in response to external pulling forces (Bustamante et al. 2004; Janshoff et al. 2000; Zhuang and Rief 2003). Here, we have demonstrated that the *apparent* dynamic visco-elastic properties of single (bio-)molecules can be extracted from the thermal motion of the AFM cantilever during force-extension experiments. The results presented are in good agreement with those recently reported (Kawakami et al. 2004, 2005) but have been acquired with a time resolution of less than 10 ms. This is at least one order of magnitude faster than other experimental approaches based on the analysis of the PSD of the cantilever motion (Kawakami et al. 2004, 2005). Since the proposed approach only requires a choice of the proper type of cantilever it can be directly applied to study protein- and RNA-unfolding and the dynamic properties of complex bio-molecular systems.

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