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Stretching a Macromolecule in an Atomic Force Microscope: Statistical Mechanical Analysis

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ABSTRACT We formulate the proper statistical mechanics to describe the stretching of a macromolecule under a force provided by the cantilever of an Atomic Force Microscope. In the limit of a soft cantilever, the generalized ensemble of the coupled molecule–cantilever system reduces to the Gibbs ensemble for an isolated molecule subject to a constant force in which the extension is fluctuating. For a stiff cantilever, one obtains the Helmholtz ensemble for an isolated molecule held at a fixed extension with the force fluctuating. Numerical examples and predictions for experiments with cantilevers of differing stiffness are given for short and long chains of poly (ethylene glycol), based on parameter-free ab initio calculations.

INTRODUCTION

A series of papers have reported the measurements of the mechanical properties of single macromolecules with the atomic force microscope (AFM) (Florin et al., 1994; Lee et al., 1994a,b; Moy et al., 1994; Rief et al., 1997a,b; Lantz et al., 1999; Ortiz and Hadziioannou, 1999; Oesterhelt et al., 1999). The experiment proceeds as follows: A macromolecule is anchored on the surface of a substrate, and the functionalized tip of an AFM cantilever picks up the molecule somewhere along its chain. By moving the cantilever, the molecule is stretched by the elastic force of the deflecting cantilever. Thus one obtains the mechanical response of the macromolecule in the form of the force–extension curve.

The force–extension relation or, in thermodynamic terms, the mechanical equation of state, can be measured and calculated under different boundary conditions: 1) One can fix the length of the macromolecule and measure the force necessary to maintain this length; this suggests doing the statistical mechanics in the isothermal–isochoric or Helmholtz ensemble in which the length is a control variable and the average force and its fluctuations are calculated by differentiation. 2) One can apply a given force and measure the resultant extension of the molecule; this suggests doing the statistical mechanics in the isothermal–isobaric or Gibbs ensemble in which the force is a control variable, and the length and its fluctuations are calculated by differentiation; a discussion is given, for instance, by Flory (1989). In the case of a one-dimensional chain, isochoric and isobaric imply constant or fixed length and force, respectively.

Because different ensembles in statistical mechanics are only equivalent for thermodynamically large systems but not for small systems in which fluctuations are non-negligible, it is important to formulate the right statistical mechanics for the stretching of a macromolecule in an AFM experiment to facilitate the correct interpretation of the experimental data and to extract the maximum amount of information from it. (This is also desirable for other, equivalent, experiments such as with laser tweezers, but will not be done here.) The question to be answered is which of the two thermodynamically conjugate variables, force and extension, is held constant and which is the fluctuating response. We will show in this paper that, in an AFM experiment, both situations can be realized by changing the force constant of the cantilever. So far, experiments were done (approximately) under the second boundary condition, mainly for reasons of sensitivity as we will discuss in detail below. Recently, a first principles theory was developed by Kreuzer et al. (1999) using both Gibbs and Helmholtz ensembles. Applied to the stretching of poly (ethylene glycol) (PEG), both in hexadecane and in water, quantitative agreement was achieved with the experimental results by Oesterhelt et al. (1999), based on the Gibbs ensemble. Different statistical mechanical ensembles for a stretched polymer have also been studied within the context of simple models (Gaussian chain and freely jointed chain) by Titanah et al. (1999).

We show a schematic of the experimental setup in Fig. 1. In the absence of contact between the cantilever tip and the macromolecule, the tip would be at a distance D from the surface where the macromolecule is anchored. When the tip is attached to the macromolecule, the latter is stretched to an end-to-end length L_m and the tip is deflected by a distance L_c such that

$$D = L_m + L_c. \tag{1}$$

Whereas L_m is always positive, L_c can have either sign. In the experiment, the distance D is adjusted and the resulting deflection, L_c, of the tip is measured optically. From this, the force is calculated assuming Hooke's law

$$F = k_c L_c, \tag{2}$$

or, if this is not valid, from an otherwise measured force law. Likewise, the extension, L_m, of the macromolecule is

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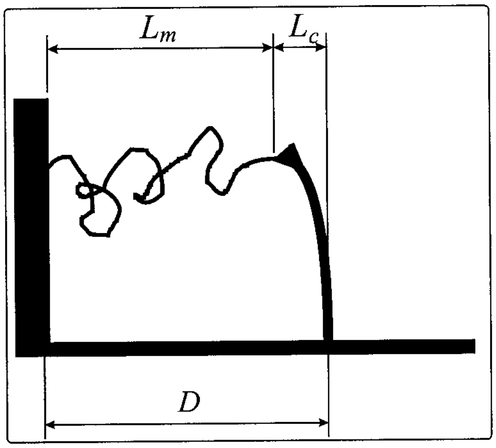

FIGURE 1 Schematic of an AFM experiment to measure the force–extension curve of a macromolecule.

calculated from Eq. 1. Obviously L_m undergoes thermal fluctuations, and, consequently, F and L_c are also fluctuating quantities.

Based on this view of the AFM experiment, we will, in the following section, develop the statistical mechanics of the coupled macromolecule–cantilever system and discuss the limiting cases of soft and stiff (compared to the macromolecule) cantilevers. In the subsequent section, we will then show numerical examples of the force–extension curves of PEG stretched with different cantilevers. The underlying potential energy curves for the various conformers of PEG will be calculated with ab initio (density functional theory) methods so that our predictions for AFM experiments on the stretching of this molecule are essentially parameter free. We also calculate Helmholtz and Gibbs potentials and entropies in addition to fluctuating quantities, stretch moduli, and segment elasticities. The paper ends with a summary of the essential insights and some conclusions.

STATISTICAL MECHANICS OF STRETCHING

In this section, we define the proper framework of statistical mechanics for the description of the stretching of a macromolecule in the atomic force microscope. We treat the tip (cantilever) and the macromolecule as two coupled subsystems whose lengths are unknown and to be measured and calculated. The experimentally controlled variables are the distance D and the temperature T. The microstates of the system are those of the two subsystems for various lengths and internal excitations. We first introduce canonical configurational partition functions of the two decoupled subsystems for given lengths, $Z_\mathrm{m}(T, L_\mathrm{m})$ and $Z_\mathrm{c}(T, L_\mathrm{c})$, where the subscripts refer to the macromolecule (m) and the cantilever (c). Coupling the two subsystems together allows the total system to sample all lengths L_m and L_c. Although the structure and the internal vibrational excitation spectrum of the macromolecule (more precisely of the relevant conformers of the macromolecule) as a function of its length must be calculated from quantum mechanics, its coupling to the cantilever can be described adequately by classical statistical mechanics because it involves only its center-of-mass motion. We can therefore write, for the total partition function

$$Z_\mathrm{system}(T, D)$$

$$= h^{-1}\int_0^\infty \mathrm{d}L_\mathrm{m}\int_{-\infty}^\infty \mathrm{d}p e^{-\beta p^2/2\mu} Z_\mathrm{m}(T, L_\mathrm{m}) Z_\mathrm{c}(T, D - L_\mathrm{m}) \tag{3}$$

$$= \lambda_\mathrm{m}^{-1}\int_0^\infty \mathrm{d}L_\mathrm{m} Z_\mathrm{m}(T, L_\mathrm{m}) Z_\mathrm{c}(T, D - L_\mathrm{m}). \tag{4}$$

Here Planck's constant accounts for the size of elementary cells in phase space, and $\beta = 1/k_\mathrm{B}T$ is the inverse temperature. Performing the integration in Eq. 3 over the momenta, p, of the center-of-mass motion of the macromolecule and of the cantilever (of reduced mass μ) we introduce the thermal wavelength $\lambda_\mathrm{m} = h/(2\pi\mu k_\mathrm{B}T)^{1/2}$.

Strictly speaking, L_m is the z-component of a vector with the z-direction along D. To restrict the cantilever to exert only stretching forces on the molecule, we could impose an upper integration limit D in Eq. 4. This is what mostly happens with long polymer chains that can easily curl up. However, short chains may resist compression so that the cantilever must be allowed to bend away from the macromolecule, and the upper limit in Eq. 3 can be much larger than D, and infinity for simplicity.

From Eqs. 3 and 4, we get the Helmholtz free energy of the total system

$$f(T, D) = -k_\mathrm{B}T \ln Z_\mathrm{system}(T, D), \tag{5}$$

which yields the average force on the system

$$\bar{F}(T, D) = \left.\frac{\partial f(T, D)}{\partial D}\right|_\mathrm{T}. \tag{6}$$

Because the coupled macromolecule–cantilever system is in internal equilibrium, this is also the force with which the cantilever acts on the macromolecule and vica versa. We get for the average length of the macromolecule

$$\bar{L}_\mathrm{m}(T, D) = \frac{\int_0^\infty \mathrm{d}L_\mathrm{m} L_\mathrm{m} Z_\mathrm{m}(T, L_\mathrm{m}) Z_\mathrm{c}(T, D - L_\mathrm{m})}{\int_0^\infty \mathrm{d}L_\mathrm{m} Z_\mathrm{m}(T, L_\mathrm{m}) Z_\mathrm{c}(T, D - L_\mathrm{m})}, \tag{7}$$

and of the deflection of the cantilever

$$\bar{L}_\mathrm{c} = D - \bar{L}_\mathrm{m}. \tag{8}$$

We get the force–extension curve of the macromolecule, i.e., $\bar{L}_{\mathrm{m}}(T, \bar{F})$, by solving Eqs. 6 and 7 simultaneously for a given temperature and varying distances D. Its explicit form obviously depends on both the intrinsic properties of the macromolecule and of the cantilever.

To make closer contact with the AFM experiment, we specify the cantilever to be well approximated by a harmonic spring with spring constant k_{c}. Its canonical partition function is

$$Z_{\mathrm{c}}(T, L_{\mathrm{c}}) = \exp[-\tfrac{1}{2}\beta k_{\mathrm{c}} L_{\mathrm{c}}^2]. \tag{9}$$

Typical cantilevers used in AFM experiments have force constants varying from 1 to 100 pN/Å. We then get for the force

$$\begin{aligned}\bar{F}(T, D) &= -\frac{k_{\mathrm{B}}T}{\lambda_{\mathrm{m}}}\frac{1}{Z_{\mathrm{system}}}\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\frac{\partial}{\partial D}\exp\left[-\frac{\beta k_{\mathrm{c}}}{2}(D - L_{\mathrm{m}})^2\right] &(10)\\ &= \frac{k_{\mathrm{c}}}{\lambda_{\mathrm{m}}}\frac{1}{Z_{\mathrm{system}}}\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})(D - L_{\mathrm{m}})Z_{\mathrm{c}}(D - L_{\mathrm{m}}) &(11)\\ &= k_{\mathrm{c}}(D - \bar{L}_{\mathrm{m}}) &(12)\end{aligned}$$

where $\bar{L}_{\mathrm{m}}$ is the average length of the macromolecule, Eq. 7. Thus, the average force is determined by measuring the average deflection $(D - \bar{L}_{\mathrm{m}})$ of the cantilever as required by Eq. 2. Note, in particular, that Eq. 12 demonstrates that both the length of the macromolecule and the force needed to maintain this length are fluctuating quantities. For these we have generally

$$\begin{aligned}(\delta L_{\mathrm{m}})^2 &= \overline{L_{\mathrm{m}}^2} - \bar{L}_{\mathrm{m}}^2\\ &= \frac{\int_0^{\infty} \mathrm{d}L_{\mathrm{m}}(L_{\mathrm{m}} - \bar{L}_{\mathrm{m}})^2 Z_{\mathrm{m}}(T, L_{\mathrm{m}})Z_{\mathrm{c}}(T, D - L_{\mathrm{m}})}{\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})Z_{\mathrm{c}}(T, D - L_{\mathrm{m}})},\end{aligned} \tag{13}$$

and, for the harmonic cantilever,

$$(\delta F)^2 = k_{\mathrm{c}}^2(\delta L_{\mathrm{m}})^2 \tag{14}$$

so that

$$\frac{\delta F}{\bar{F}} = \frac{\delta L_{\mathrm{m}}/\bar{L}_{\mathrm{m}}}{D/\bar{L}_{\mathrm{m}} - 1}. \tag{15}$$

To further clarify the force–extension relation, we write in Eq. 9

$$\begin{aligned}L_{\mathrm{c}}^2 = (D - \bar{L}_{\mathrm{m}})^2 + 2(D - \bar{L}_{\mathrm{m}})(\bar{L}_{\mathrm{m}} - L_{\mathrm{m}})\\ + (\bar{L}_{\mathrm{m}} - L_{\mathrm{m}})^2,\end{aligned} \tag{16}$$

and insert this in Eq. 7 to get

$$\bar{L}_{\mathrm{m}} = \frac{\int_0^{\infty} L_{\mathrm{m}}\mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\exp[\beta\bar{F}L_{\mathrm{m}}]\exp[\mathscr{A}]}{\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\exp[\beta\bar{F}L_{\mathrm{m}}]\exp[\mathscr{A}]}, \tag{17}$$

where

$$\mathscr{A} = -\frac{\beta k_{\mathrm{c}}}{2}(\bar{L}_{\mathrm{m}} - \bar{L}_{\mathrm{m}})^2.$$

The last exponential function in both numerator and denominator involves the length fluctuations. The simultaneous solution of Eqs. 12 and 17 yields the force–extension curve for a macromolecule stretched by a harmonic spring cantilever. Importantly, at this stage, this force–extension curve depends not only on the intrinsic properties of the macromolecule, via $Z_{\mathrm{m}}(T, L_{\mathrm{m}})$, but also on the elastic properties of the cantilever via its harmonic force constant k_{c}. However, what one aspires to measure in the AFM (or any other) experiment are the intrinsic properties of the macromolecule. We will show now that the effect of the cantilever can be minimized (essentially eliminated) from the measurements by judicious choices of the cantilever properties, namely either very soft or very stiff cantilevers.

Soft cantilever

To simplify Eq. 17 for a soft cantilever, we take the limits

$$k_{\mathrm{c}} \rightarrow 0 \qquad D \rightarrow \infty$$
$$k_{\mathrm{c}}D = \text{const.} \tag{18}$$

The last condition ensures that the average force remains nonzero. This reduces Eq. 17 to

$$\bar{L}_{\mathrm{m}} \simeq \frac{\int_0^{\infty} L_{\mathrm{m}}\mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\exp[\beta\bar{F}L_{\mathrm{m}}]}{\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\exp[\beta\bar{F}L_{\mathrm{m}}]}. \tag{19}$$

This is exactly the expression one would write for the average length using the Gibbs (or isothermal–isobaric) ensemble of an isolated macromolecule to which an external force is applied whose origin is not explicitly identified, i.e., from a Gibbs partition function and Gibbs potential

$$Z_{\mathrm{m}}^{(\mathrm{Gibbs})}(T, F) = \lambda_{\mathrm{m}}^{-1}\int_0^{\infty} \mathrm{d}L_{\mathrm{m}} Z_{\mathrm{m}}(T, L_{\mathrm{m}})\exp[\beta F L_{\mathrm{m}}], \tag{20}$$

$$g(T, F) = -k_{\mathrm{B}}T \ln Z_{\mathrm{m}}^{(\mathrm{Gibbs})}(T, F), \tag{21}$$

$$\bar{L}_{\mathrm{m}} = -\left.\frac{\partial g(T, F)}{\partial F}\right|_{\mathrm{T}}. \tag{22}$$

(The sign convention in Eqs. 20–22 has the mechanical energy increasing by $F\,\mathrm{d}L_{\mathrm{m}}$ for a displacement $\mathrm{d}L_{\mathrm{m}}$ of the macromolecule.) The only difference is that, using the Gibbs ensemble, one explicitly assumes that the external force is experimentally controlled and thus does not fluctuate. Indeed, we can demonstrate this point for a soft cantilever explicitly by looking at the force fluctuations. The limit, Eq. 18, implies that $D/\bar{L}_m \rightarrow \infty$ so that, from Eq. 15, we see that the force fluctuations become arbitrarily small. This is exactly the prerequisite for the use of the Gibbs ensemble for the macromolecule. Thus the criterion for a very soft cantilever is that $D/\bar{L}_m \gg 1$. This is indeed the case in the series of experiments done by Gaub and his coworkers. There is of course a balance to be struck in the sense that the noise in the cantilever increases with its softness.

Stiff cantilever

To examine the case of a stiff cantilever, we start from the system partition function Eq. 4 and note that, in the limit,

$$k_c \rightarrow \infty, \tag{23}$$

the cantilever partition function, Eq. 9, approaches a delta function

$$Z_c(T, L_c) = \exp\left[-\frac{1}{2}\beta k_c(D - L_m)^2\right] \rightarrow \left(\frac{2\pi}{\beta k_c}\right)^{1/2} \delta(D - L_m). \tag{24}$$

We get, for the partition function,

$$Z_{\text{system}}(T, D) \rightarrow Z_m(T, D)\frac{k_B T}{\hbar\omega_c}, \tag{25}$$

and for the free energy,

$$f_{\text{system}}(T, D) \rightarrow f_m(T, D) - k_B T \ln(k_B T/\hbar\omega_c). \tag{26}$$

Here, $\omega_c = (k_c/m)^{1/2}$ is the frequency, increasing with increasing stiffness of the cantilever, at which the center of mass of the macromolecule oscillates in response to the force exerted by the rigid cantilever. In Eq. 26, the first term is the Helmholtz free energy of the isolated macromolecule with fixed length D, and the second term arises from the cantilever. This term does not approach zero as k_c approaches infinity because we have used classical statistics in Eq. 4 with the inherent assumption that $k_B T/\hbar\omega_c \gg 1$. This cosmetic blemish can easily be remedied by using quantum statistics throughout. We have not done this here because AFM stretching experiments are done at room temperature with big molecules justifying the use of classical statistics.

With Eq. 26, the force, Eq. 6, becomes

$$\bar{F}(T, D) \simeq -k_B T \frac{1}{Z_m(T, D)} \left.\frac{\partial Z_m(T, D)}{\partial D}\right|_T. \tag{27}$$

This is precisely what we would have written if we had started with an isolated macromolecule with length D specified, and thus worked in the Helmholtz ensemble (for the isolated macromolecule rather than for the coupled macromolecule–cantilever system) with T and D the natural variables. That this is indeed the case can be demonstrated by observing that the limit, Eq. 23, implies that $D/\bar{L}_m \rightarrow 1^+$. Rewriting Eq. 15 as

$$\frac{\delta L_m}{\bar{L}_m} = (D/\bar{L}_m - 1)\frac{\delta F}{\bar{F}}, \tag{28}$$

we see that, in this limit, the length fluctuations are reduced to zero, which is the prerequisite for the use of the Helmholtz ensemble for the (isolated) macromolecule. Our criterion for a very stiff cantilever is, therefore, that $(D/\bar{L}_m - 1) \ll 1$. This limit is experimentally more difficult to achieve than the soft limit in that the deflection of a stiff cantilever is obviously very small so that its sensitivity becomes poor. Yet, as we will see in the numerical examples in the next section, this limit is physically also interesting and thus worth pursuing experimentally. One should keep in mind that, once theory has produced a quantitative explanation of the soft cantilever experiments, it is an easy task to calculate what one would expect for a stiff cantilever.

NUMERICAL EXAMPLES

To calculate the force–extension curve for a given macromolecule, we must first obtain its canonical partition function, $Z_m(T, L_m)$, for a fixed length L_m. Next we must specify the force constant, k_c of the cantilever. Last, for a range of D values, we determine $\bar{L}_m(T, \bar{F})$ by solving Eqs. 7 and 12 selfconsistently. We will do this for two systems: a short chain of PEG with only three subunits, and a longer chain with 21 subunits.

A short chain of PEG

We have recently presented a theoretical description (Kreuzer et al., 1999; H. J. Kreuzer and M. Grunze, submitted for publication) of the force measurements reported by Oesterhelt et al. (1999) on individual PEG chains in different solvents, i.e., PBS buffer or Hexadecane. In our first principles theory, we calculated the energy spectrum (or the density of states) for PEG chains from quantum mechanics, and used the Gibbs ensemble to derive the force–extension curve.

The first task is to calculate the energy spectrum of oligo (ethylene oxide) as a function of chain length for a given number of EG units; here EG stands for (–O–CH_2–CH_2–). Because ab initio quantum mechanical calculations of the electronic structure of a molecule scale at least with the fourth power of the number of electrons, one has to restrict oneself to rather small molecules, in our case, $CH_3(EG)_3OCH_3$. As we have shown, this is not a serious restriction for a quantitative description of the late stages of the elastic response, essentially because the total response of a long chain is more or less additive over the individual EG units, provided the ab initio calculations are done as accurately as possible for all the conformers of the (small) molecule. Whereas, in the previous paper (Kreuzer et al., 1999), we based our calculations on the Hartree–Fock method (at the MP2/6-31++G**//HF/3-21G level), we have, in the meantime, switched to density functional theory (also using the GAUSSIAN 98 suite of programs, Frisch et al., 1998) using the option BP86/6-311++G** for the exchange/correlation potential. The same stability series for the various conformers was obtained as with the best MP2 calculations, with most energies and geometries very close, to within better than ten percent. See H. J. Kreuzer and M. Grunze (submitted for publication) and, for more details, Wang et al. (2000).

The various conformers of $(EG)_3$ form local minima on the total energy surface. Of these, we have selected 27 according to the following criteria. In the EG subunit (–O–CH_2–CH_2–) all the C–O bonds are kept in the trans configuration. (We have also calculated a few conformers with gauche rotations around C–O bonds. They are typically higher in energy, but of similar length, than those with a *trans* configuration, and thus contribute little to the force–extension curve.) This information can then be omitted from the notation, so that, as an example, the helical and all-*trans* conformers, (tg^+t–tg^+t–tg^+t) and (ttt–ttt–ttt), can be denoted as ($g^+g^+g^+$) and (ttt), respectively. We thus have three EG units, g^+, g^-, and t, to be placed on three positions along the chain, or $3^3 = 27$ combinations or conformers. To calculate their structure, we start from a conformer with standard C–C and C–O bond lengths (1.52 and 1.42 Å), C–C–O and C–O–C bond angles (109° and 112°) and dihedral angles (±74°, 180°), but adjust all these parameters, and also allow rotation around the C–C bond to find a local minimum in the total electronic energy. Because these calculations are done for isolated molecules, their symmetry implies that there are only ten energetically different groups of conformers, e.g., $E(g^+g^+g^+) = E(g^-g^-g^-)$, $E(g^+g^+g^-) = E(g^-g^+g^+) = E(g^+g^-g^-) = E(g^-g^-g^+)$, etc. Here the energy E is the total electronic energy of the conformer. In Fig. 2, we show the potential energy curves, $V_i(L_m)$, for these ten (energetically different) conformers. Further details of the calculations are given by Wang et al. (2000) where we also discuss solvation effects.

Having the potential energy curves for all the conformers as a function of their lengths, we can write down the canonical partition function of the isolated macromolecule

$$Z_m(T, L_m) = \sum_{i=1}^{10} g_i \exp[-\beta V_i(L_m)] \prod_k z_k^{(i)}(L_m). \qquad (29)$$

The sum over i runs over all ten conformers with each term multiplied by the degeneracy g_i of that particular conformer, i.e., the number of other conformers with the same energy as listed in the caption of Fig. 2. The product over k exhausts all the internal vibrational/rotational modes of each conformer with $z_k^{(i)}(L)$ the corresponding partition function. The internal modes, for the most part, do not change drastically as a function of the length of the conformer, so that their contribution to the force–extension curves is usually negligible, but they contribute to the free energy and entropy.

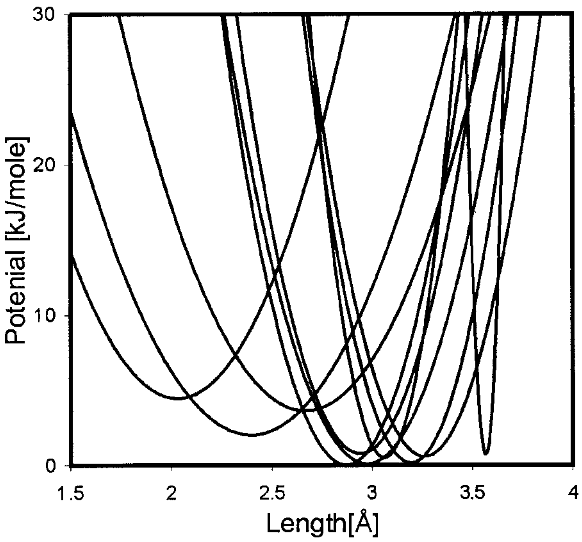

FIGURE 2 Potential energy curves of ten conformers of $(EG)_3$ as a function of the end-to-end length per monomer. From left to right according to the minimum (degeneracy in front): $2(g^+g^-g^+)$, $4(g^+g^+g^-)$, $4(g^+g^-t)$, $2(g^+g^+g^+)$, $4(g^+g^+t)$, $2(g^+tg^+)$, $2(g^+tg^-)$, $2(tg^+t)$, $4(g^+tt)$, 1(ttt).

In Fig. 3, we show force–extension curves for tri-(ethylene oxide) as stretched by cantilevers for a range of spring constants. The calculations proceed as follows. We select a specific setting of D, as in the AFM experiment, and the length $\bar{L}_m(T, D)$ is calculated using Eqs. 7, 9, and 29. The selfconsistent force $\bar{F} = \bar{F}(T, \bar{L}_m)$ for a macromolecule stretched by a harmonic spring cantilever follows from Eq. 12. Repeating this procedure for a sequence of D-settings, a full force–extension curve is recorded. How much this curve deviates from the true force–extension curve of an isolated macromolecule depends on the force constant of the cantilever, as is clear from the sequence of panels in Fig. 3.

In the top panel of Fig. 3, we show the force–extension curves for soft cantilevers. For spring constants less than 10 pN/Å, these curves are, to within a fraction of a percent, equal to those of an isolated macromolecule stretched by an external force as calculated with the Gibbs ensemble as discussed above. In the same panel, we can also see that lowering the temperature from 300 to 100 K sharpens up some features in the force–extension curve. We have also plotted the settings of D necessary to measure this curve with a cantilever of 10 pN/Å. Not surprisingly, this weak cantilever needs substantial deflection (about three times the length of the macromolecule at maximum extension) to produce forces of the order of 400 pN. Large deflection of

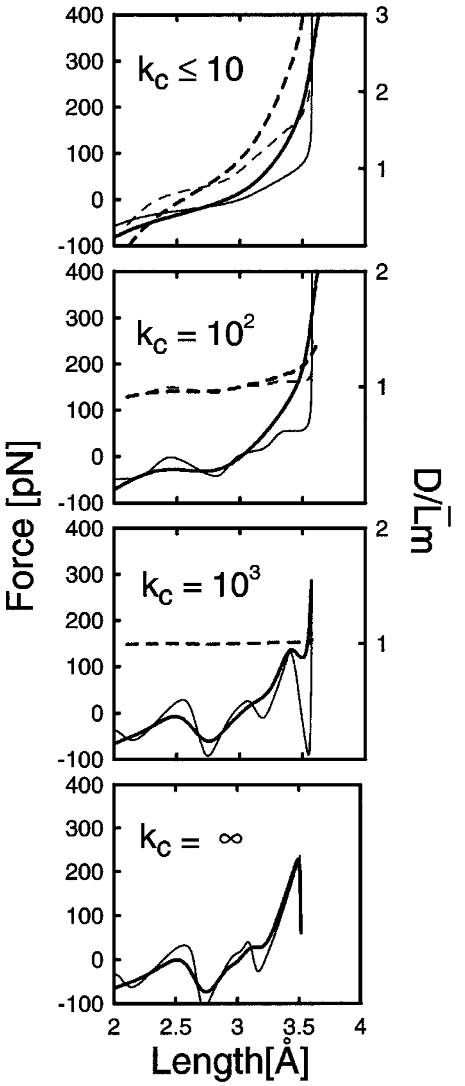

FIGURE 3 Force–extension curves for $(EG)_3$ as measured in an AFM experiment with cantilevers of different force constants k_c in picoNewton per Angstrom as indicated at 300 K (*thick solid lines*) and at 100 K (*thin solid lines*). Also shown as dashed lines is the ratio of the position D of the cantilever to the length $\bar{L}_m$ of the macromolecule (*right scale*).

course also implies good sensitivity of the cantilever provided its fluctuations remain manageable.

In the two center panels of Fig. 3, we show the force–extension curves (again for 300 and 100 K) for larger force constants, showing already a substantial modification from the soft cantilever (i.e., Gibbsian) limit. We emphasize that, in an AFM experiment with intermediate strength cantilevers (for $(EG)_3$ this is the range from 10^2 to 10^3 pN/Å), the measured force–extension curve would not be that of an isolated macromolecule. It would take considerable effort (such as our theoretical approach presented here) to disentangle the features arising from the macromolecule itself and those from its coupling to the cantilever. Also note that, for these cantilevers, the D settings needed are only slightly larger than the end-to-end length of the stretched macromolecule, i.e., the deflections of the cantilever are already marginal making their experimental detection difficult.

In the bottom panel of Fig. 3, we show force–extension curves for two temperatures as calculated from Eq. 27 for a very (infinitely) stiff cantilever. These curves correspond to the first boundary condition in the Introduction, namely that a distance D is fixed between the ends of the macromolecule and the external force necessary to keep the clamp at that position is measured. These curves, albeit measured under different limits for the cantilever, contain information solely about the intrinsic properties of the isolated macromolecule, just as the ones in the top panel of Fig. 3, measured under the Gibbsian boundary conditions of the Introduction.

To see the physical significance of the different boundary conditions, i.e., fixed length (Helmholtz) versus fixed force (Gibbs), it is useful to evaluate Eq. 27 for the partition function, Eq. 29. We get

$$\bar{F}(T, L_m) = \frac{1}{Z_m(T, L_m)} \sum_{i=1}^{10} g_i \left[\frac{dV_i(L_m)}{dL_m} - k_B T \sum_{l} \left. \frac{\partial \ln z_l^{(i)}(L_m)}{\partial L_m} \right|_T \right] \exp[-\beta V_i(L_m)] \prod_k z_k^{(i)}(L_m). \quad (30)$$

The first term implies that the average force is obtained by taking the derivatives of the potential energy curves of all the conformers at a given length L_m and weighting them with their Boltzmann factors. At low temperatures, this implies that only the energetically lowest conformer contributes at a given length so that the force switches from negative to positive as one proceeds from left to right of the minimum of one conformer. In contrast, if we measure the force–extension curve at fixed force, we sample all conformers that have a slope corresponding to the specific force at which the measurement is made. These two situations are illustrated schematically for three conformers in Fig. 4. At fixed length, we take the derivatives of the three potential energy curves and add these different forces with their respective Boltzmann factors. At fixed force, we sample those points on the potential energy curves at different lengths where the derivatives are the same.

Ideally, in an experiment, one would switch to cantilevers with larger force constants for the measurement of the high-force regime to minimize the cantilever extension $D - \bar{L}_m$ and reduce its fluctuations.

A long chain of PEG

One can use the results of ab initio calculations for short polymer molecules to construct an interacting Ising-like chain model valid for any length of the polymer. By necessity, one looses some details, both structurally and energet-

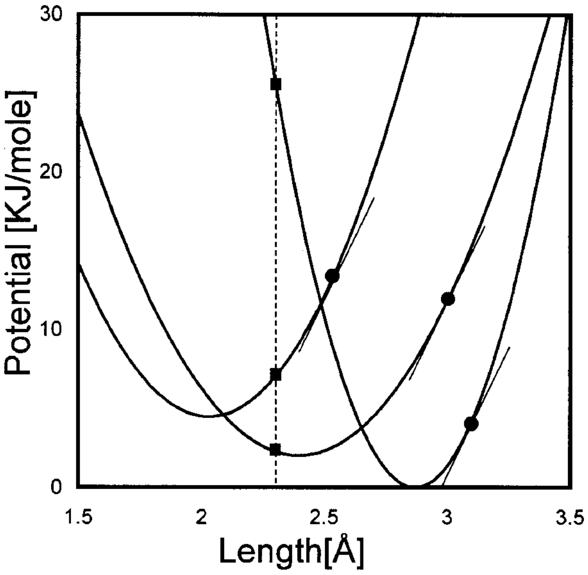

FIGURE 4 Schematic to illustrate the different boundary conditions. Working at fixed length (Helmholtz ensemble), one samples different forces, i.e., the derivatives of the potential energy curves at that length (*squares*). Working at fixed force, one samples the same slope on the energy curves, albeit at different lengths (*circles*).

ically, but it turns out that this loss is far compensated by the advantages of being able to deal with large molecules.

In an interacting chain model of PEG, we allow individual EG units to be in three independent conformations, ttt, tg$^+$t, and tg$^-$t; their designation we abbreviate to t, g$^+$, and g$^-$. We define occupation number vectors, $\mathbf{n}_i$, whose transpose takes values $\mathbf{n}_i^T = (100)$, (010), and (001) if the *i*th EG unit along the chain is t, g$^+$, or g$^-$, respectively. We also define a vector of self-energies $\mathbf{E} = (E_t, E_g, E_g)$ and a matrix of nearest-neighbor interaction energies,

$$\overleftrightarrow{\mathbf{V}} = \begin{pmatrix} V_{tt} & V_{tg} & V_{tg} \\ V_{tg} & V_{gg} & V_{g^+g^-} \\ V_{tg} & V_{g^+g^-} & V_{gg} \end{pmatrix}. \tag{31}$$

We then write the hamiltonian of a chain of N EG units as

$$H(\mathbf{n}_1, \mathbf{n}_2, \ldots, \mathbf{n}_N) = E_s + \sum_{i=1}^{N} \mathbf{E} \cdot \mathbf{n}_i + \sum_{i=1}^{N-1} \mathbf{n}_i^T \cdot \overleftrightarrow{\mathbf{V}} \cdot \mathbf{n}_{i+1}. \tag{32}$$

E_s is the energy of the terminating groups at each end of the chain. The self energies, E_t and E_g, the nearest-neighbor interactions V_{tt}, V_{tg}, etc., are determined from the ab initio calculations of short $(EG)_n$ chains.

To obtain the geometry of a given conformer, we must specify bond lengths and angles. We do this for the EG units as a whole rather than for the individual C–C and C–O bonds within a EG unit. We proceed by listing all the unit lengths of the g and t units in all the conformers of $(EG)_3$ and then take the averages. Similarly, we look at all the bond angles between gg, gt, g$^+$g$^-$, and tt neighboring pairs in all the conformers and again determine their averages. Last, we determine the dihedral angles in all the conformers of $(EG)_3$. Details of these calculations and all the parameters of the chain model will be given elsewhere (L. Livadaru, R. R. Netz, and H. J. Kreuzer, manuscript in preparation).

Using this interacting chain model, we have calculated the force extension curves for PEG with 21 EG subunits for various force constants of the cantilever. In the top panel of Fig. 5, we show the results for a soft cantilever with $k_c = 1$ pN/Å; the force–extension curves do not change for softer cantilevers. Because longer chains have significantly more conformers and thus are much more flexible, the force is positive down to end-to-end lengths of less than 1 Å per

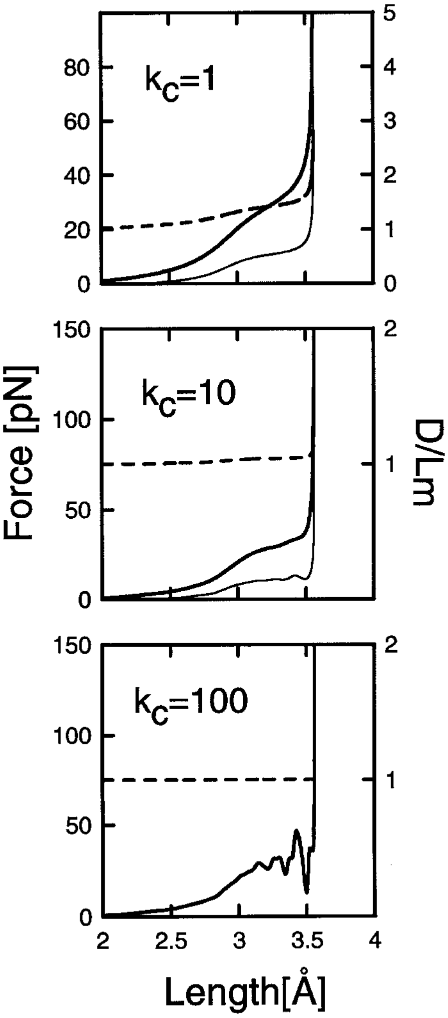

FIGURE 5 Force–extension curves for $(EG)_{21}$ as measured in an AFM experiment with cantilevers of different force constants k_c in picoNewton per Angstrom as indicated, at 300 K (*thick solid lines*) and at 100 K (*thin solid lines*). Also shown, as dashed lines, is the ratio of the position D of the cantilever to the length $\bar{L}_m$ of the macromolecule (*right scale*).

monomer, cf. Fig. 3. Remarkable is the change in the distance D needed for the measurement of the force–extension curves as one goes from a short to a long chain, and also from stiffer to softer cantilevers. Whereas for the short chain and $k_c = 1$ pN/Å, we need $D/\bar{L}_m \simeq 20$ for the largest extension, this ratio is less than 2 for the long chain, but would go up to 10 for $k_c = 0.1$ pN/Å. In contrast, for a long chain and $k_c = 10$ pN/Å (*center panel* of Fig. 5), the setting D is only 10–20% larger than $\bar{L}_m$ for the largest extension. These numbers agree remarkably well with the settings in the experiment by Oesterhelt et al. (1999).

In the bottom panel of Fig. 5 we finally show the force–extension curve for a stiff cantilever. Again, as for the shorter chain, we are approaching the Helmholtz limit in which the force starts to oscillate between attraction and repulsion as different conformers contribute. This is most pronounced at the largest extension (and also at lower temperatures) where the number of conformers available becomes rather sparse. Note also that, with the cantilever much stiffer than the molecule itself (except for the longest extension possible, where within an interacting chain model the stiffness is infinite), the setting D is only marginally larger than the resulting extension of the molecule, $\bar{L}_m$.

FLUCTUATIONS AND THERMODYNAMICS

Next we look at the fluctuations of the length of the macromolecule around its mean, $\delta L_m/\bar{L}_m$, shown in Fig. 6 for $(EG)_3$. For a soft cantilever, there is a substantial variation of the fluctuations as the macromolecule is stretched, decreasing to minimal values as the maximum extension of the molecule is approached. This is shown for a force constant $k_c = 10$ pN/Å at 300 K (*thick solid line*) and with more structure at 100 K (*thin solid line*); the dashed-dot line is for the Gibbs limit (vanishingly soft cantilever). For a stiff cantilever, $k_c = 10^3$ pN/Å, the fluctuations are much smaller overall and show less dependence on the extension of the macromolecule (*dashed lines*).

To extend these results to longer chains, such as for PEG with 750 EG units, we can estimate the relative fluctuations by scaling with the square root of the ratio of the respective lengths, $\sqrt{3/750} = 0.06$, yielding $\delta L_m/\bar{L}_m < 0.003$ in the high force regime. In contrast, we can estimate these fluctuations from the force fluctuations using Eq. 14. From the experiments on PEG by Oesterhelt et al. (1999) we estimate $\delta F \approx 10$ pN so that, for $k_c = 10$ pN/Å, we have $\delta L_m/\bar{L}_m \approx 0.0004$, and for $k_c = 1$ pN/Å we have $\delta L_m/\bar{L}_m \approx 0.004$, in reasonable agreement with the theoretical estimate.

We have also calculated the segment elasticity per monomer,

$$K_s = N \frac{\partial \bar{F}}{\partial \bar{L}_m}\bigg|_T, \tag{33}$$

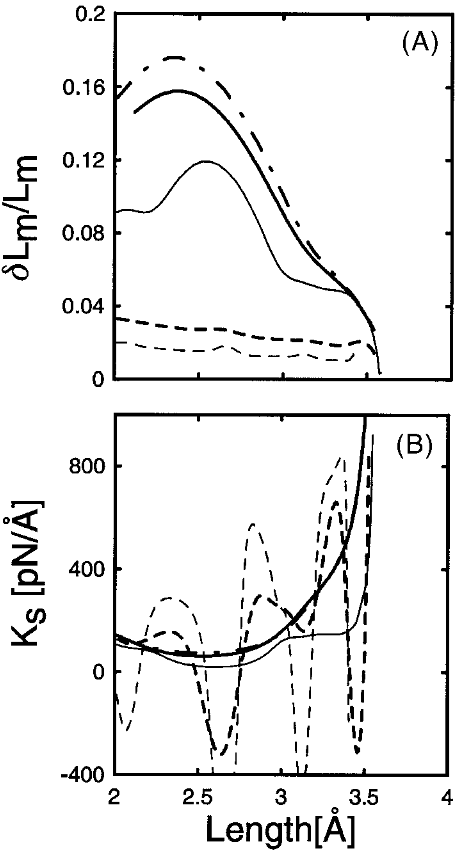

FIGURE 6 Relative length fluctuations (*a*) and segment elasticity (*b*) of $(EG)_3$ when coupled to cantilevers of different force constants. *Dashed lines*, $k_c = 10^3$ pN/Å; *solid lines*, $k_c = 10$ pN/Å; and *dashed-dot line*, $k_c = 0$; *thick lines*, 300 K; and *thin lines*, 100 K.

which is plotted in the lower panel of Fig. 6 for $(EG)_3$ for stiff (*dashed lines*) and soft (*solid lines*) cantilevers at two temperatures. In the Helmholtz limit (stiff cantilever) the segment elasticity has obviously much more structure reflecting the structure in the force–extension curve. In contrast, in the Gibbs limit (soft cantilever), the segment elasticity is fairly constant (~100–150 pN/Å) up to the regime of extreme forces, i.e., close to bond rupture where it increases dramatically.

For completeness, we mention that we can also relate the segment elasticity to the length fluctuations

$$K_s = N\left[\frac{k_B T}{(\delta L_m)^2} - k_c\right] \tag{34}$$

$$= N\left[\frac{k_B T/\bar{L}_m^2}{(\delta L_m/\bar{L}_m)^2} - k_c\right]. \tag{35}$$

Thus, although the relative length fluctuations are smallest for a stiff cantilever, their variation with $\bar{L}_m$ is important.

Last, in Fig. 7, we show the Helmholtz free energy of the macromolecule–cantilever system, Eq. 5, and the entropy

$$s(T, D) = -\left.\frac{\partial f(T, D)}{\partial T}\right|_{D} \tag{36}$$

plotted as a function of the average length of the macromolecule, for the cantilever force constants and temperatures of Fig. 3, excluding the contributions from the internal vibrational modes of the macromolecule. (The internal vibrational modes arise from 84 degrees of freedom for $(EG)_3$ and contribute at 300 K and zero force ~255 k_BT to the free energy and 29 k_B to the entropy (Wang et al., 2000).) For a soft cantilever (*top panel*) the Helmholtz free energy of the total system is rather structureless and the Gibbs free energy (as a function of the average length and not of its proper variable, the force), $g(T, \bar{L}_m(D)) = f(T, D) - \bar{F}D$, looks like the Gibbs free energy of the isolated macromolecule. For a stiff cantilever (*third panel*) the Helmholtz free energy of the total system follows the envelope of the conformer potential energy curves, particularly at low temperature, and is thus very similar to the Helmholtz free energy for the isolated macromolecule (*bottom panel*) from which one can obtain the force–extension curve by direct differentiation.

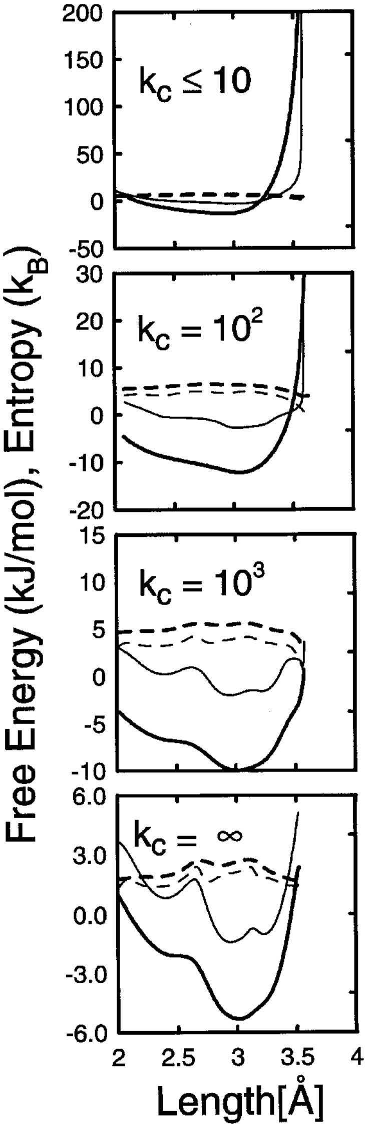

FIGURE 7 Helmholtz free energy (*solid lines*) and entropy (*dashed lines*) of $(EG)_3$ when coupled to cantilevers of different force constants as in Fig. 3. Thick lines at 300 K and thin lines at 100 K.

The entropies are fairly constant for soft and moderately stiff cantilevers at ~5–7 k_B, because of the fact that the conformer potentials overlap, see Fig. 2. Structure only shows up in the Helmholtz limit where, for very low temperatures, the entropy is the logarithm of the sum of the degeneracies of the contributing conformers, e.g., it is $k_B \ln 2$ around the minimum of the $(g^+g^-g^+)$ conformer, $k_B \ln 4$ around the minimum of $(g^+g^+g^-)$, and it develops a narrow peak of height $k_B \ln(4 + 2)$ where these two potentials cross, and similarly for the other conformers. An example of such behavior has been published previously by Kreuzer et al. (1999).

SUMMARY

In this paper, we have set up the theory to describe the stretching of a macromolecule by a cantilever in an AFM experiment. We have shown that, for intermediate cantilever force constants, the elastic and energetic properties of both the macromolecule and the cantilever contribute to the force–extension curve in an intricate way, roughly describable as a convolution. However, for soft cantilevers, a situation can be achieved in which the effect of the cantilever on the force–extension curve becomes negligible, and the latter can be calculated using the Gibbs ensemble for an isolated molecule, as we have done in our previous work (Kreuzer et al., 1999; H. J. Kreuzer and M. Grunze, submitted for publication). In contrast, for very stiff cantilevers the force–extension curve resembles that which one would obtain from a calculation in the Helmholtz ensemble of the isolated macromolecule. These two ensembles do not produce the same mechanical equation of state (i.e., force–extension curve) as they would for a macroscopically large system, because polymer molecules even with several hundred monomer units are still substantially influenced by fluctuations, in particular, in the force needed to stretch them.

As examples of this theory and in an attempt to understand quantitatively the experiments by Oesterhelt et al. (1999) on the stretching of PEG, we have used, as input, the energetics and structure of the conformers as calculated with ab initio methods. The resulting force–extension curves are thus parameter-free and relevant as predictions for experiments where the cantilever compliance can be varied. Where a comparison is already possible, namely for long PEG molecules stretched with a soft cantilever, the agreement is excellent.

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