Correction of the viscous drag induced errors in macromolecular manipulation experiments using atomic force microscope

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We describe a method to correct the errors induced by viscous drag on the cantilever in macromolecular manipulation experiments using the atomic force microscope. The cantilever experiences a viscous drag force in these experiments because of its motion relative to the surrounding liquid. This viscous force superimposes onto the force generated by the macromolecule under study, causing ambiguity in the experimental data. To remove this artifact, we analyzed the motions of the cantilever and the liquid in macromolecular manipulation experiments, and developed a novel model to treat the viscous drag on the cantilever as the superposition of the viscous force on a static cantilever in a moving liquid and that on a bending cantilever in a static liquid. The viscous force was measured under both conditions and the results were used to correct the viscous drag induced errors from the experimental data. The method will be useful for many other cantilever based techniques, especially when high viscosity and high cantilever speed are involved. © 2010 American Institute of Physics. [doi:[10.1063/1.3436646](https://doi.org/10.1063/1.3436646)]

I. INTRODUCTION

The atomic force microscopy (AFM) and related techniques have been increasingly used to study biological macromolecules at the single molecule level. These experimental studies have established a number of facts on the structural properties and functional mechanisms of nucleic acids, proteins, and polysaccharides.^{1–5} The AFM uses a microcantilever to exert and measure forces on the macromolecule that is tethered between a substrate surface and a tip at the end of the cantilever. Most of these macromolecular manipulation experiments are performed in buffer solutions, where the force applied to the macromolecule is controlled by moving the cantilever relative to the substrate surface. During such measurements, the cantilever experiences a viscous drag force due to its motion in the solution. This viscous force superimposes onto the force generated by the macromolecule under study, causing ambiguity in the measured forces and difficulties in data interpretation. The viscous drag effect becomes significant when the speed of the cantilever is high⁶ or the viscosity of the solution is high such as in the study of macromolecular crowding effects on the mechanical stability of proteins.⁷

To determine this viscous drag effect on the cantilever and correct the associated artifacts in the experimental data, several methods have been reported.^{6,8} The essence of these methods is to use the scaled spherical model to treat the cantilever as a sphere-spring system and use Stokes' law to determine the viscous force. The theory for the hydrodynamic drag on a sphere in a semi-infinite space of uniform flow under low Reynolds number conditions predicts that the viscous force is dependent on the relative speed, the size of the sphere, the sphere-surface separation, and the liquid viscosity.^{9,10} From the measured viscous drag force at various liquid speeds and tip-surface separations, parameters

characterizing the shape and size of the cantilever are obtained by fitting the data to the theoretical model. These parameters are then used to correct the errors caused by viscous drag in the experimental data obtained with the same type of cantilevers.⁶ These methods have provided a reasonable estimate of the viscous drag on the AFM cantilevers; however, there is a fundamental deficiency in these approaches because the cantilever's mode of motion during the viscous force determination is different from that during a macromolecular manipulation experiment. As shown in Fig. 1(a), in a mechanical protein unfolding experiment, the polypeptide chain is tethered between the AFM tip and a surface.² As the surface is moved away from the cantilever, the liquid around the cantilever travels with approximately the same speed as the surface,^{6,8} and the cantilever is bent by forces from the molecule as well as from the viscous drag. As depicted in the figure, the viscous drag force is not distributed uniformly along the cantilever since the relative speed of the liquid with respect to the cantilever is higher at the fixed end than that at the free end. When an unbinding or unfolding event occurs in the macromolecule, the free end of the cantilever will move in the opposite direction as the surface, leading to a higher relative liquid speed at the free end than that at the fixed end, as shown in Fig. 1(b). In contrast, the viscous drag force measurement in the previously reported methods⁶ was made when the cantilever was bent into a static conformation by the liquid as shown in Fig. 1(c); consequently, the viscous force is distributed uniformly along the cantilever. Such a measurement does not accurately reflect the hydrodynamic processes as shown in Figs. 1(a) and 1(b). The viscous force thus measured is not equivalent to that experienced by the cantilever in a macromolecular manipulation experiment for the same tip speed relative to the liquid.

In the work reported here, we model the cantilever as a rotating beam and treat the viscous drag on the cantilever in

FIG. 1. Schematics of the cantilever motion and associated viscous force (F_{vis}) distribution in different experimental settings. (a) In an experiment of mechanical unfolding of proteins, as the protein is pulled, the cantilever is bent downward by forces from the extensible polyprotein chain and from the viscous drag. The tip moves in the same direction as the liquid but at a lower speed (the liquid moves at the same speed as the surface), resulting in a nonuniform viscous force distribution as shown. (b) When an unfolding event occurs, the cantilever recoils toward its equilibrium position, and the tip moves in the opposite direction as the liquid, resulting in a viscous force distribution as shown. (c) In a reported method of measuring the viscous force (Ref. 6), the cantilever is bent into a static conformation by the liquid motion, and the viscous force distribution is uniform. (d) The bending motion of the cantilever is modeled as the rotation of a rigid beam with a length of $2L/3$. The shape of the cantilever (gray line) was calculated using Eq. (1). In the figure, θ is the angle between the bent model cantilever and the equilibrium (undeflected) position, d is the cantilever deflection, s is the tip-surface separation, and D is the distance between the surface the equilibrium tip position.

macromolecular manipulation experiments as a superposition of the viscous force on a static cantilever in a moving liquid and that on a bending cantilever in a static liquid. Measurements were made to determine the viscous force on the cantilever under both hydrodynamic conditions, yielding parameters that are amenable to the experimental conditions as those shown in Figs. 1(a) and 1(b). These parameters are then used to correct the viscous force induced errors in the experimental data of mechanical unfolding of proteins.

II. MODELING THE CANTILEVER MOTION IN LIQUID

When a rectangular cantilever is bent by a tethered molecule as shown in Fig. 1(a), the shape is described by the function¹¹

$$z = \frac{Fy^2}{2kL^3}(y - 3L), \quad (1)$$

where z is the displacement of the cantilever from the equilibrium position as a distance y from the fixed end [see Fig. 1(d)], F is the force acting on the tip, L is the length, and k is the force constant of the cantilever. The bending of a V-shaped cantilever can be treated as a rectangular cantilever following the parallel beam approximation.¹² In order to elu-

cidate the viscous drag force on the cantilever, we model the cantilever as a beam of length L with $L/3$ fixed and the other $2L/3$ being able to rotate about the point at $L/3$, as shown in Fig. 1(d). For the same amount of deflection d , the cantilever and the model beam have the same slope at the free end, and the areas swept by them are also similar (differing by $\sim 10\%$). Since the cantilever deflection in an AFM is measured by its slope at the free end, and the viscous drag on the cantilever is directly related to the area swept within a specific time period, the rotating beam as shown in Fig. 1(d) is a reasonable model for the bending cantilever. A similar treatment has been used to describe the bending of a nanofilament.¹³

When the beam rotates in a liquid that is also moving, the equation of motion is

$$I\ddot{\theta} = \int_0^{2L/3} (\gamma_l v_{\text{liquid}} - \gamma_c \dot{\theta} y) dy - k\theta(2L/3)^2 + F_{\text{ext}}(2L/3), \quad (2)$$

where I is the momentum of inertia, k is the force constant of the cantilever, and F_{ext} is the externally applied force at the tip. The first term on the right-hand side is the torque due to viscous drag, the second term is the torque of the restoring force, and the last term is the torque of an external force. To determine the torque of the viscous drag in Eq. (2), the viscous force experienced by an element dy of the beam is written as the superposition of the viscous force on a static cantilever by the moving liquid and that on a moving cantilever in a static liquid (see Fig. 1): $dF_{\text{vis}} = \gamma_l v_{\text{liquid}} dy - \gamma_c v_{\text{cant}} dy$, where v_{liquid} and $v_{\text{cant}} \equiv y\dot{\theta}$ are the speeds of the liquid and the cantilever (at location y), respectively, and γ_l and γ_c are proportional constants. In solving Eq. (2), the left-hand side is taken to be zero since the inertia term is negligible for the AFM cantilever moving in liquid (Reynolds number $\sim 10^{-3}$).^{8,14} With the initial condition of $\theta_{t=0} = \theta_0$, the solution is

$$\theta = \frac{\gamma_l v_{\text{liquid}}}{2k} + \frac{3F_{\text{ext}}}{2kL} + \left(\theta_0 - \frac{\gamma_l v_{\text{liquid}}}{2k} - \frac{3F_{\text{ext}}}{2kL} \right) e^{-(9k/2\gamma_c L)t}. \quad (3)$$

The deflection of the beam is given by

$$\begin{aligned} d &= \frac{2L}{3}\theta = \frac{\gamma_l v_{\text{liquid}} L}{3k} + \frac{F_{\text{ext}}}{k} - \frac{4\gamma_c L}{27k} \dot{\theta} \\ &= \frac{\gamma_l v_{\text{liquid}} L}{3k} + \frac{F_{\text{ext}}}{k} - \frac{4\gamma_c L^2}{9k} \ddot{d}. \end{aligned} \quad (4)$$

Therefore, when the cantilever deflects in a viscous liquid, the apparent end-loading force is

$$F = kd = (b_l v_{\text{liquid}} - b_c v_{\text{tip}}) + F_{\text{ext}}, \quad (5)$$

where $v_{\text{tip}} \equiv \dot{d}$ is the linear speed of the tip, $b_l \equiv \gamma_l L/3$, and $b_c \equiv 2\gamma_c L/9$. Equation (5) shows that the effect of the viscous drag due to the liquid motion, which is uniformly distributed along the cantilever, is equivalent to an end-loading force of $b_l v_{\text{liquid}}$, while the viscous drag due to the cantilever bending (tip motion), which is not uniformly distributed (Fig. 1), is equivalent to an end-loading force of $-b_c v_{\text{tip}}$. In

our approach, the coefficients of viscous drag b_l and b_c are experimentally measured and the values are then used to remove the viscous drag induced artifact in the experimental data.

III. MEASURING THE COEFFICIENTS OF VISCOUS DRAG

Measurements were made on two types of cantilevers from Veeco Instruments: a rectangular cantilever (length = 200 μm , width = 20 μm , and thickness = 0.6 μm) and a V-shaped cantilever (length = 180 μm , width = 18 μm , and thickness = 0.6 μm). The force constants of the cantilevers are individually determined by thermal fluctuation analysis.¹⁵ A commercial AFM (Nanoscope IIIa, Digital Instruments/Veeco) that has been modified for force measurements was used.¹⁶ At the beginning of an experiment, the AFM tip is positioned at a desired distance (in the range of 0.3–1.2 μm) from the sample surface (gold coated mica) without the tethered molecule. The surface was then moved away from (or toward) the tip at a specified speed (in the range of 8–32 $\mu\text{m/s}$) for a distance of 20 nm. Within this distance, the cantilever was bent by the viscous force due to the motion of the liquid around it, which moved at (approximately) the same speed as the surface.⁶ For all the liquids used, the cantilever reached a maximum bending, where v_{tip} became zero, within the 20 nm distance. After the surface motion (thus the liquid motion) was stopped, the cantilever bounced back to its equilibrium position with a time-dependent deflection given by Eq. (3) [inset, Fig. 2(a)]. The cantilever deflection signal was recorded during both the viscous drag induced deflection and the recoil processes. Forty measurements were made for each set of parameters: the tip-surface distance, the surface speed, and the liquid viscosity.

Figure 2 shows the cantilever deflection as a function of time obtained in different liquids. From these curves, the value of b_l was obtained from the maximum cantilever deflection d using Eq. (5) with $F_{\text{ext}}=0$, $v_{\text{tip}}=0$, and v_{liquid} equal to the surface (pulling) speed. The value of b_c was found by fitting the time-dependent cantilever deflection after stopping the liquid motion to the exponential function of Eq. (3), with $F_{\text{ext}}=0$, $v_{\text{liquid}}=0$, and the deflection $d=(2L/3)\theta$. The measured values of b_l and b_c in a 50% (v/v) glycerol/water solution for both cantilevers are shown in Fig. 3 as a function of the tip-surface separation. Identical results for the values of b_l and b_c were obtained with the surface moving away from the tip (cantilever bending downward) and with surface moving toward the tip (cantilever bending upward). After corrections for the differences in cantilever sizes and solution viscosities, the values of b_l as those shown in Fig. 3 are consistent with the results reported by Janovjak *et al.*⁶ and Alcaraz *et al.*,⁸ where measurements were made on cantilevers that had been bent to a steady state ($v_{\text{tip}}=0$). It can be seen from Fig. 3 that the values of b_c and b_l are clearly different for the same cantilever. Therefore, when both the cantilever and the liquid are in motion [Figs. 1(a) and 1(b)], the viscous force should be calculated using Eq. (5): $F_{\text{vis}}=(b_l v_{\text{liquid}}-b_c v_{\text{tip}})$. The viscous drag force will be overesti-

FIG. 2. (Color online) Cantilever deflection as the sample surface was moving downward at a constant speed for the first half of the measurement period, and then stopped. In each figure, the horizontal straight line is a fit to the maximum cantilever deflection and the solid curve on the right-hand side is a fit to the exponential function in Eq. (3). The coefficients of the viscous drag b_l and b_c are obtained from the fits using Eqs. (3)–(5). (a) The V-shaped cantilever ($k=50$ pN/nm) measured in a solution of 50% (v/v) glycerol/water, with a surface speed of 8.0 $\mu\text{m/s}$ and a tip-surface separation of 0.73 μm . The schematic diagrams in the figure show the three stages of cantilever motion during one cycle of measurements: the cantilever bends downward by viscous force as the surface moves away from the tip; the cantilever reaches the maximum deflection; and the surface stops moving and the cantilever recoils back toward its equilibrium position. The arrows indicate the directions of motion of the cantilever and the surface. (b) The rectangular cantilever ($k=20$ pN/nm) measured in 25% (v/v) glycerol/water with a surface speed of 16 $\mu\text{m/s}$ and a tip-surface separation of 0.22 μm . The values of b_l and b_c determined from the data are 4.8 and 4.6 pN s/ μm , respectively.

mated or underestimated (depending on the direction of the cantilever motion) if it is calculated only using b_l and the liquid motion relative to the tip: $F'_{\text{vis}}=b_l(v_{\text{liquid}}-v_{\text{tip}})$.⁶ Figure 4 shows the coefficients of viscous drag b_l and b_c for both cantilevers as a function of the solution viscosity. The magnitudes of these coefficients are linearly dependent on the solution viscosity, but are independent of the solution

FIG. 3. (Color online) The coefficients of viscous drag plotted as a function of the tip-surface separation. The measurements were carried out in solution of 50% (v/v) glycerol/water. The solid lines are fits to the scaled spherical model: $b=6\pi\eta a^2/(s+s_0)$ (Ref. 8), where s is the tip-surface separation, and a and s_0 are fitting parameters.

FIG. 4. (Color online) The coefficients of viscous drag plotted as a function of the solution viscosity. Measurements were made in three solutions: water, 25% (v/v) glycerol/water, and 50% (v/v) glycerol/water, with viscosities of 1.0, 2.4, and 8.6 cP, respectively. The two values of a coefficient at the same viscosity were measured under two different liquid speeds.

speed at which they are measured, suggesting that the modeling and approximations used in the measurements are reasonable.

IV. REMOVING THE VISCOUS EFFECT FROM EXPERIMENTAL DATA

Figure 5 demonstrates the correction of the viscous drag effect from a force versus extension curve, obtained from a mechanical unfolding experiment using the protein titin domain I27.¹⁷ The experiment was carried out in a solution containing a high concentration of the macromolecular crowding agent dextran,⁷ which significantly raised the viscosity of the solution. The procedure started with the measurements of the coefficients b_l and b_c for the cantilever as described above at different tip-sample distances. Using these two coefficients, the liquid speed and the tip speed, the viscous force on the cantilever was then determined at each

FIG. 5. (Color online) Correction of the viscous drag effect from experimental data of mechanical unfolding of protein molecules [Figs. 1(a) and 1(b)]. The force curve (thick solid line) was obtained from pulling an octamer of titin domain I27 in a solution containing 300 g/l dextran ($M_w = 6000$ Da, solution viscosity = 10 cP) with a pulling speed of $5 \mu\text{m/s}$. Each peak in the regular sawtooth pattern corresponds to the unfolding of a single protein, while the high peak in the beginning is due to nonspecific interaction between the tip and the sample, and the last peak comes from the detachment of the protein from the tethering surfaces. After obtaining the slope of the force curve at each point, the tip speed was calculated using Eq. (7), as shown by the thin solid line. The viscous force on the cantilever was calculated at each point, and then subtracted from the force curve. The dashed line is the force curve after the correction, which is similar to the expected unfolding forces for the protein in the crowded solution (Refs. 7 and 17).

point of the force curve according to Eq. (5): $F_{\text{vis}} = b_l v_{\text{liquid}} - b_c v_{\text{tip}}$, which was subsequently subtracted from the data. While the liquid speed, which is equal to surface speed or the pulling speed, is set by the experimenter and remains constant during a measurement, the speed and the direction of the tip motion are not constant, and have to be determined from the force curve. As can be seen from Fig. 1(d),

$$v_{\text{tip}} = \frac{\Delta d}{\Delta t} = \frac{\Delta(F/k)}{\Delta t} = \frac{1}{k} \frac{\Delta F}{\Delta s} \frac{\Delta s}{\Delta t}, \quad (6)$$

where $F = kd$ is the measured force, k is the force constant of the cantilever, and s is the tip-surface separation, which is equal to the extension of the tethered molecule [see Fig. 1(a)]. The tip-surface separation is equal to the distance traveled by the surface (D) minus the amount of cantilever deflection: $s = D - d$; therefore, $\Delta s / \Delta t = \Delta D / \Delta t - \Delta d / \Delta t = v_{\text{pull}} - \Delta d / \Delta t$, with v_{pull} being the surface (pulling) speed. Combining this with Eq. (6), we get

$$v_{\text{tip}} = \frac{\text{slope}}{k + \text{slope}} v_{\text{pull}}, \quad (7)$$

where $\text{slope} \equiv \Delta F / \Delta t$ is the slope of the force curve at the point where the viscous force is calculated. The tip speed at every point of the force curve can thus be calculated. In Fig. 5, the tip speed corresponding to the force curve is also shown. Once the tip speed is known, the viscous force on the cantilever at every point of the force curve is determined and subtracted from the force curve. The dashed line in Fig. 5 shows the force curve after such a correction. It can be seen that the unfolding forces of the protein were underestimated by about 15% due to the viscous drag effect. This result shows the necessity to remove this artifact in order to correctly interpret the experimental data obtained in viscous liquids.

V. CONCLUSIONS

Due to the complicated nature of the liquid and cantilever motions in an AFM liquid chamber, an analytical solution to the viscous drag problem is not currently available. Our model and experimental approach add physical insights to the hydrodynamics process and provide a simple method to properly remove the viscous drag effect from experimental data of single molecule manipulation of macromolecules. The method can be easily modified for AFMs of different designs as well as other techniques utilizing cantilevers. As the AFM and other cantilever based nanomanipulation techniques continue to advance, they will be used to probe a more extensive range of properties and functions on a wider variety of macromolecules. Consequently, experimental conditions involving high speeds and high viscosities will be encountered more frequently, and correction of the viscous drag induced artifact will become an indispensable part in these experiments.

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