Force microscopy imaging of individual protein molecules with sub-pico Newton force sensitivity

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The capability of atomic force microscopes (AFM) to generate atomic or nanoscale resolution images of surfaces has deeply transformed the study of materials. However, high resolution imaging of biological systems has proved more difficult than obtaining atomic resolution images of crystalline surfaces. In many cases, the forces exerted by the tip on the molecules (1–10 nN) either displace them laterally or break the noncovalent bonds that hold the biomolecules together. Here, we apply a force microscope concept based on the simultaneous excitation of the first two flexural modes of the cantilever. The coupling of the modes generated by the tip–molecule forces enables imaging under the application of forces (∼35 pN) which are smaller than those needed to break noncovalent bonds. With this instrument we have resolved the intramolecular structure of antibodies in monomer and pentameric forms. Furthermore, the instrument has a force sensitivity of 0.2 pN which enables the identification of compositional changes along the protein fragments. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: atomic force microscopy; picoNewton forces; antibodies; flexural modes; nonlinear dynamics; IgG; IgM

Received 19 June 2007; revised 2 August 2007; accepted 3 August 2007

INTRODUCTION

Progress on spatial resolution has driven the evolution of force microscopy from static to dynamic excitation modes (Bustamante and Keller, 1996; Engel and Müller, 2000; San Paulo and Garcia, 2000; Hörber and Miles, 2003; Hinterdorfer and Dufrene, 2006). In all the cases, the improvement of the spatial resolution was accompanied by a reduction of the normal and lateral forces exerted by the tip on the surface. However, achieving molecular resolution images of biological systems has been much more difficult than obtaining atomic resolution images of crystalline surfaces (Ohnesorge and Binnig, 1993; Klinov and Magonov, 2004; Giessbil and Quate, 2006). In most of the cases, the forces exerted by the tip on the molecules (1–10 nN) either displace them laterally or break the noncovalent bonds that hold the biomolecules together (Fritz et al., 1995; San Paulo and Garcia, 2000; Pignataro et al., 2002; Moreno-Herrero et al., 2003; Solares, 2007), which in turn prevents high resolution imaging. The individual noncovalent bonds that hold together the tertiary structure of proteins (∼50 pN) may be broken by the AFM probe. Furthermore, protein unfolding requires forces in the 50–100 pN range. These facts have prevented the observation by AFM of individual biomolecules in a true non-invasive manner.

Typical AFM images of individual proteins either in air or in solution are hard to interpret because they show featureless globular structures. Several attempts have
Proksch, 2006) and electrostatic interactions (Stark et al., 2007).

In this study, we demonstrate that the spatial contrast and force sensitivity of an atomic force microscope (AFM) while imaging individual biomolecules can be improved by operating the instrument at lower forces. This is achieved by operating the AFM under the simultaneous excitation of the first two cantilever modes. The root mean square (rms) amplitude of the first mode is used as the feedback parameter while the phase shift of the second mode is used to obtain compositional maps with very high force sensitivities. The tip–molecule forces generate higher harmonics of the first mode. Whenever the frequency of a higher harmonic matches or is close to the frequency of the second mode (here, $f_2 = 6.2f_1/n$, with $n = 6$) the modes become coupled. This coupling is substantially enhanced by the initial non-zero amplitude of the second mode which leads to operate the AFM at larger tip–molecule separations, i.e. under very small forces.

MATERIALS AND METHODS

Force microscopy

The experiments were performed in air at a relative humidity of 40% with a modified AM-AFM that enables the simultaneous excitation of the first and second resonances of the microcantilever (Martinez et al., 2006). The bi-modal AFM excitation force is

$$F_{ext}(x,t) = F_1 \cos \omega_1 t + F_2 \cos \omega_2 t$$

where $\omega_1$ and $\omega_2$ are the angular frequencies of the first and second resonances respectively (Figure 1A and B). The phase and amplitude of both modes are measured independently by using two quadrature circuits. The rms amplitude of the first mode is fed back to the controller for conventional topography imaging while the phase shifts of the second mode are recorded and plotted to obtain compositional maps. Imaging has been performed with free amplitudes in the range of 5–25 nm and 0.2–1 nm for the first and second mode respectively ($A_{01}$ and $A_{02}$). The feedback loop actuates on the amplitude of the first mode and it is set approximately at 0.9$A_{01}$. The tip was scanned across the surface at 1 Hz. Topography and compositional images were obtained with rectangular silicon cantilevers with nominal tip radii of 2–5 nm, force constants about 10 N/m and resonance frequency about 120 kHz (SEIHR, Nanosensors, Germany). The noise level in the force sensor for the bi-modal AFM is 70 pm/√Hz.

Antibodies

Monoclonal IgG antibodies (Ab 38C2, Sigma) samples were prepared from a stock solution of 1 mg/ml by diluting to 10 μg/ml in distilled water. A drop of 10 μl was deposited for 30 s on a freshly cleaved mica surface, rinsed in distilled water and dried with N gas. Pentameric IgM samples were prepared from purified human IgM (Sigma) by diluting the original solution 1:100 in distilled water. A drop of 10 μl was deposited for 45 s on a freshly cleaved mica surface, rinsed in distilled water and dried with N gas.

Dynamic AFM simulations

In AM-AFM there is no practical experimental protocol to measure instantaneous forces and to determine force sensitivities. This is in contrast with respect to contact AFM. Nonetheless, numerical simulations could be used to determine the forces. The simulations are performed by modelling the three dimensional microcantilever as a rectangular beam and applying the Euler–Bernoulli equation...
We also consider that the continuous beam is excited under the bi-modal regime. The model only considers long-range attractive forces between tip and sample surface. The results of the simulations are compared with experimental phase versus separation curves. The good qualitative and quantitative agreement between theory and experiments allows us to estimate the force sensitivity under the experimental conditions described here.

**EXPERIMENTAL RESULTS**

Antibodies are proteins that have well defined structures (Hafner et al., 1999; Alberts et al., 2002; Kienberger et al., 2004) and binding sites (Kienberger et al., 2004) which make them good candidates to test the sensitivity and resolution of the bi-modal AFM for biomolecular imaging. Immunoglobulin G (IgG) is a Y-shaped protein that consists of four polypeptide chains arranged in three fragments, one Fc receptor and two identical Fab antigen-binding sites (Figure 2D). The van der Waals length of each fragment is about 6.5 nm. Because of the flexibility of IgG, the antibody adopts several morphologies and in particular the Y shape.

We have used the bi-modal AFM to image small and flexible IgG antibodies deposited on mica. Figure 2A shows a second mode phase image of IgG antibodies, in particular two of them have the Y-shaped morphology. It has been shown before that the antibodies can adopt different orientations on the support (San Paulo and Garcia, 2000; Kienberger et al., 2004). Figure 2B shows an individual antibody with Y-shaped orientation with all its fragments accessible to AFM image analysis. The cross-section along the lines depicted in Figure 2B allows us to identify the position of the Fc and Fab fragments (Figure 2C). Two of the fragments show the same phase shift of 5° while the third fragment shows a phase shift at 8° (Figure 2C). The phase shifts are relative to the mica surface. This result where the phase shift on the molecule shows three peaks, two of them are identical and smaller in value than the other, has also been found on other IgG molecules. Consequently, we assign the largest peak to the Fc fragment and the smaller peaks to the Fab fragments. The result indicates that the identification of different components along the protein chains is possible by the present method.

We have also imaged complex protein structures such as immunoglobulin M pentamers (IgM) (Figure 3A). IgM has five Ig monomers, each of them having one Fc and two Fab fragments. Additionally there is a small polypeptide chain (J-chain) joining two consecutive Fc fragments. The image shows several IgM molecules together with smaller structures that could be detached fragments of IgM. The pentagonal shape and the position of the J-chain (central structure) are readily recognized in high resolution bi-modal phase images (Figure. 3B–D). We have measured the angles between the lines joining the J-chain and two consecutive monomers. The histogram shows a single and dominant peak centred at 70°, i.e. very close to the theoretical value of 72° for a rigid pentamer (Figure 3E). The above difference as well as the slightly distorted pentagonal structure observed in Figure 3B–D are attributed to the flexible character of the...
Fab fragments. Previously, the only AFM images of IgM that hinted its pentagonal structure were obtained by using sharp carbon nanotube probes (Hafner et al., 1999).

A direct comparison between amplitude modulation (tapping mode) and bi-modal AFM images illustrates some of the advantages of the latter for high resolution imaging of isolated biomolecules under the application of very small forces. To assure a meaningful comparison, we have used an AFM that enables to perform both amplitude modulation and bi-modal AFM imaging with the same cantilevers. Figure 4A and B show tapping mode topography and phase shift image of an IgM antibody. The image was obtained by applying a maximum tip–molecule force below 100 pN. The small value of the force explains the lack of contrast in both AM-AFM images (see discussion below). Figure 4C shows the bi-modal AFM phase image obtained along with Figure 4A and B. The bi-modal AFM image shows the five Ig monomers as well as the J-chain. Similar results have been obtained by imaging IgG molecules. Tapping mode AFM images of a single IgG in both topography and phase shift (Figure 4D and E) show a featureless and faint object while the bi-modal AFM image resolves the Fab and Fc fragments of the protein (Figure 4F).

The spatial resolution in probe microscopy is defined as the minimum separation between two objects for which the dimple depth is larger than the instrumental noise (Bustamante and Keller, 1996). This criterion applied to Figures 2 and 3 where Fc, Fab, J-chain and hinge regions are resolved, gives a resolution of 1.5 nm.

THEORETICAL SIMULATIONS

The force sensitivity under bi-modal AFM operation is estimated by numerical simulations. The simulations are performed by modelling the three dimensional cantilever with a rectangular beam and solving the Euler–Bernoulli equation of motion under bi-modal excitation (Rodriguez and Garcia, 2004). The dynamic deflection function \( w(x,t) \) of the cantilever follows

\[
EI \frac{\partial^4}{\partial x^4} \left[ w(x,t) + a_1 \frac{\partial w(x,t)}{\partial t} \right] + bh_0 \frac{\partial^2 w(x,t)}{\partial t^2} = F(x,t)
\]

(2)
where \( x \) is the normalized coordinate along the beam and \( L \) the cantilever length; \( b \) and \( h \) are the width and thickness while \( E \) is the Young modulus, \( I \) the moment of inertia, \( \rho_c \) is the mass density and \( a_1 \) is the internal damping of the cantilever. The boundary conditions assume one end of the cantilever clamped and the other free. The relevant forces, expressed in the above equation in units of force per length, are the external excitation, hydrodynamic damping with the medium and tip–sample interaction forces respectively:

\[
F(x, t) = F_{\text{ext}}(x, t) + F_{\text{med}}(x, t) + F_{\text{ts}}(x, t)
\]  

(3)

The excitation force includes first and second mode components (see Equation 1). The model might include both

Figure 4. (A) Amplitude modulation AFM image (tapping mode) of IgM molecule (topography). (B) Amplitude modulation phase shift image of (A). (C) Bi-modal AFM phase image of the same IgM pentamer. The image shows the five subunits and the central structure (J-chain). Figure 4A–C have been obtained simultaneously. (D) Amplitude-modulation AFM topography image of an IgG molecule. (E) Phase shift image corresponding to (D). (F) Bi-modal AFM phase image of the same IgG. The image reveals the presence of Fab and Fc fragments. Figure 4D–F have been obtained simultaneously (\( A_{01} = 23 \) nm, \( A_{02} = 0.4 \) nm).
F\textsubscript{ts}(X = L) = - \frac{\alpha}{d^2} \tag{4}

where \(\alpha\) represents the strength of the interaction. In particular, for van der Waals forces the strength of the interaction can be expressed by \(\alpha = HR/6\), where \(H\) is the Hamaker constant and \(R\) the effective interaction radius. The time-dependence of the Euler–Bernoulli equation was solved numerically by using a fourth-order Runge–Kutta algorithm.

Figure 5A shows the dependence of the second mode phase shift with respect to the oscillation amplitude (which is equivalent to the average tip–surface distance). The experiment is performed by approaching the vibrating tip towards the mica surface from a distance (mean value) several times larger than the free amplitude \(A_0\). The experimental curve shows three regions, two regions characterized by high slopes at separations of \((0.7–1)A_0\) and \((0–0.15)A_0\) respectively, and a transition region with a nearly constant slope between 0.16 and 0.7\(A_0\). The quantitative agreement obtained between experiment and theory supports the validity of the model to describe bi-modal AFM operation. The above agreement allows us to calculate the force exerted by the tip on the sample and the force sensitivity of the second mode phase signal by fitting experimental data to simulations. We remark that in AM-AFM there are no direct experimental measurements of the maximum force exerted on the sample.

The maximum force \((F_{\text{max}})\) exerted by the probe on the molecule is calculated by using the range of parameters for which a good fit between theory and experiments exists. Figure 5B shows the dependence of the \(F_{\text{max}}\) on the amplitude ratio. The force curve depends on the initial free amplitudes (filled circles \(A_0 = 24\text{ nm}\) versus open circles \(A_0 = 6\text{ nm}\)). For relevant experimental values \((A_0\in[5\text{ and } 20\text{ nm}]\) and \(A_0/\text{AC}[0.8 \text{ and } 1]\)), the force varies between 1 and 100\(p\text{N}\). For small free amplitudes (open circles) the force initially increases by decreasing the amplitude, and then it flattens out. At smaller amplitude ratios it increases again. The experimental phase shift profile of the second mode shown in Figure 2D gives a maximum phase shift of 7\({}^\circ\). The simulations show that the above phase shift profile involves maximum forces in the 35–38\(p\text{N}\) range (Figure 5C). From the above curve, we obtain an approximate value of the slope \(\approx 0.7\text{ pN/degree}\). For a bandwidth of 3\(kHz\) (imaging bandwidth) the noise in the phase signal is \(\Delta \phi \approx 0.3^\circ\) which translates into \(\delta F \approx 0.2\text{ pN}\), i.e. about two orders of magnitude smaller than previously reported by any AFM at the same bandwidth.

**DISCUSSION AND CONCLUSIONS**

The enhanced sensitivity obtained by the bi-modal AFM is a consequence of the nonlinear coupling of the first two flexural resonances, the higher sensitivity of the second mode with respect to the first, the use of phase shifts instead of amplitudes to detect forces and because we have decoupled microscopy operation from force detection. In AM-AFM operation, average tip–surface force and contrast are intrinsically linked by the expression (San Paolo and Garcia, 2001),

\[
(F_{\text{ts}}) = \frac{k}{2Q} \sqrt{|A_0^2 - A^2|/2} \tag{5}
\]
where \( k \) and \( Q \) are respectively the static force constant and the quality factor of the fundamental mode (first resonance). The typical tapping mode operation parameters are \( k = 20–50 \text{ N/m} \), \( A_0 = 5–10 \text{ nm} \), \( A = 0.7 \) \( A_0 \) and \( Q \) is approximately 200. Substituting those values in Equation 5 yields the average tip–sample force to be in the range 100–900 pN. In bi-modal AFM, the first mode tracks the topography in exactly the same way as in AM-AFM. Equation 5 for the experimental parameters used in Figures 2–4 gives an average tip–sample force of 190 pN (\( A = 0.9 \) \( A_0 \)). That force is higher than the forces given by solving Equation 1 (~50 pN). The latter difference could be traced back to the fact that the point-mass model used to derive Equation 5 cannot capture the existence of higher eigenmodes. The tip–surface adhesion forces prevent the use of smaller free amplitudes. Thus, to operate the microscope under smaller forces will require the set-point amplitudes very close to \( A_0 \). This in turn implies to withdraw the tip with the subsequent reduction of the signal to noise ratio and loss of spatial resolution (see for example tapping AFM images taken at 0.9\( A_0 \) in Figure 4B and E). Bi-modal AFM overcomes the above limitation by using the second mode as an additional interaction channel. The nonlinear coupling between first and second mode makes the latter extremely sensitive to short and long-range tip–surface interactions because it has both a higher quality factor (\( Q_2 = 5Q_1 \)) and a higher optical sensitivity in beam deflection detectors (~4:1). Furthermore, the amplitude of the second mode is very small (\( A_{02} = 0.5 \text{ nm} \)) which enables this mode to explore the relevant range of the interaction potential. In addition, the feedback loop does not actuate on the amplitude of the second mode so its phase shift instantly responds to minute tip–molecule force variations.

In summary, we have resolved the intramolecular structure of antibodies in monomer and pentameric forms by using a force microscope concept that involves the simultaneous excitation of its first two eigenmodes. We have demonstrated that under the same conditions, bi-modal AFM gives higher spatial contrast and resolution than tapping mode AFM. We have also demonstrated that bi-modal AFM allows to perform imaging in air under the application of maximum forces below 100 pN. We have estimated that the force sensitivity of this force microscopy mode is 0.2 pN. Finally, we conclude that the identification of the protein subunits based on measurements which are directly related to the strength of the tip–surface forces and thus to the chemical composition would enable this technique to image large protein complexes with true non-invasive forces.

Acknowledgments

This work was financially supported by the European Commission (FOR-CEETOOL, NMP4-CT-2004-013684) and the Ministerio de Educacion y Ciencia (MAT2006-03833).

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