Accurate Wide Modulus Range Nanomechanical Mapping of Ultrathin Interfaces with Bimodal Atomic Force Microscopy

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ABSTRACT. Nanoscale determination of the mechanical properties of interfaces is of paramount relevance in materials science and cell biology. Bimodal AFM is arguably the most advanced nanoscale method to map the elastic modulus of interfaces. Simulations, theory and experiments have validated bimodal AFM measurements on thick samples (from μm to mm). However, the bottom-effect artifact, this is, the influence of the rigid support on the determination of the Young's modulus questions its accuracy for ultrathin materials and interfaces (1-15 nm). Here, we develop a bottom-effect correction method that yields the intrinsic Young's modulus value of a material with independence of its thickness. Experiments and numerical simulations validate the accuracy of the method for a wide range of materials (1 MPa-100 GPa). Otherwise, the Young's modulus of an ultrathin material might be overestimated by a 10-fold factor.

KEYWORDS

Nanomechanics, bimodal AFM, elastic modulus, ultrathin layers, lipid bilayers

INTRODUCTION

Nanoscale mechanical property mapping is one of the key achievements of atomic force microscopy (AFM).¹⁻⁵ Nanomechanical mapping is applied to facilitate the development or to improve the characterization of materials and interfaces.⁶⁻¹⁵ Nanomechanical measurements are widely used in mechanobiology to reveal how elastic and viscoelastic properties of proteins, cells and the extracellular matrix influence biological processes.¹⁶⁻²²

A variety of AFM-based methods have been developed over the last 25 years to map mechanical properties at the nanoscale. Among them, force volume^{1,3-4}, AFM phase imaging², bimodal AFM^{4,23}, torsional harmonics,²⁴⁻²⁵ contact resonance²⁶⁻²⁸ or multifrequency AFM.²⁹ The accuracy of some of those methods on thick samples, notably the two most widely applied, force volume (force-distance curve methods) and bimodal AFM have been validated by performing comparisons with macroscopic techniques, finite-element methods and numerical simulations. However, some key applications in materials science and mechanobiology might involve the nanomechanical characterization of ultrathin materials (1-20 nm) such as nanostructured materials, 2D materials, lipid membranes, DNA or proteins deposited on rigid supports.

Dimitriadis, Chadwick and co-workers³⁰ showed that the elastic modulus of the rigid support influences the AFM measurement on cells. This finding led to the so-called bottom effect artifact which, in turn, have stimulated different correction models to address the determination of elastic and viscoelastic properties measured by force volume (AFM-based force-distance curves).³¹⁻³⁴ Bottom-effect corrections were also proposed for multilayer materials.^{33, 35-37} Several of the corrections were numerical solutions which were difficult to implement to process AFM experiments.

Bimodal AFM is arguably the fastest and most versatile method to map nanomechanical properties of surfaces and interfaces.^{23,38} It has been applied to characterize the elastic properties of polymers,³⁹⁻⁴⁰ hybrid materials,⁴¹ inorganic and organic crystals,⁴²⁻⁴³ self-assembled

monolayers,⁴⁴ lipid membranes,⁴⁵ DNA,^{41,46} proteins,⁴⁷⁻⁵⁰ and cells.⁵¹ It combines high-spatial resolution (angstrom or nanoscale) with quantitative accuracy on the determination of the Young's modulus. Recently, the method has been extended to measure adhesion forces at the nanoscale.⁵²

A variety of theories and simulations have supported and/or demonstrated the capabilities of bimodal AFM.⁵³⁻⁶³ Currently, it is the only method that provides nanomechanical maps at 5 frames per second (high speed).⁵⁰ Despite its widespread use, the accuracy of the bimodal nanomechanical measurements performed on ultrathin materials might be jeopardized by the bottom-effect artifact.^{4,30}

Here, we develop a bottom-effect correction method for bimodal elastic property measurements performed on ultrathin layers and nanostructured materials. Numerical simulations show that the analytical expression enable to recover the Young's modulus of ultrathin layers with a relative error below 5 % over a 5-order of magnitude modulus range (1 MPa-100 GPa). The method is applied to determine Young's modulus of supported lipid bilayers immersed in liquid. Bimodal AFM provided a Young's modulus value of 13 MPa which was independent on the number of lipid bilayers. Force volume measurements performed on the same sample provided a similar value.

RESULTS AND DISCUSSION

Figure 1 shows a scheme of bimodal AFM nanomechanical measurement performed on ultrathin materials. Figure 1a shows the tip-layer-solid support interface while Fig. 1b describes some parameters of bimodal AFM. Specifically, we have applied the bimodal AM-FM configuration. In this configuration, the amplitude of the first mode is kept constant while the phase shift of the second mode is locked at 90° with respect to its driving force.^{48,64}

Bottom effect correction theory for bimodal AFM. In bimodal AFM, the procedure to determine elastic modulus from the observables is well established. It involves the application of the virial theorem to excited modes.^{48,53} This leads to two independent equations, one for each excited mode,

$$V_i = \langle F_{ts} z_i \rangle = \frac{1}{T} \int_0^T F_{ts}(z) z_i dt \quad (1)$$

where z_i are the deflection of the excited modes and z is the total cantilever deflection ($z \approx z_1 + z_2$). In many situations of interest, T is well approximated by the period of the first eigenmode ($T \approx T_1$).^{50, 52} The above equation can be solved by two independent approaches. First, by using integrating the equation of motion of the excited modes. It has been shown that for $(A_{01} \gg A_{02})$ ^{48,53}

$$V_{1m} = \frac{k_1 A_1 A_{01}}{2Q_1} \cos \phi_1 \quad (2)$$

$$V_{2m} = \frac{k_2 \Delta f_2}{f_2} A_2^2 \quad (3)$$

We have used the subscript m to indicate that the virials are directly determined (measured) from the observables $(A_1, A_2, \phi_1, \Delta f_2)$. In the above equations k_1 , Q_1 , A_1 and A_0 are respectively the force constant, quality factor, instantaneous amplitude and free amplitude of the 1st eigenmode; k_2 , A_2 , f_2 and Δf_2 are respectively the force constant, instantaneous amplitude, resonant frequency and resonant frequency shift of the 2nd eigenmode. Second, the virials might be independently determined by solving the integral in eq 1 with a F_{ts} force given by a contact mechanics model.

In a previous contribution,⁴⁸ we deduced an analytical expression to determine the Young's modulus E_s based on a semi-infinite model. The expression was deduced by assuming that the force exerted by a paraboloid of radius R on a material (with independence of its thickness) was described as a function of the indentation I by the Hertz (Sneddon-paraboloid) model⁶⁵

$$F_{Hertz} = \frac{4}{3} E \sqrt{R} I^{3/2} \quad (4)$$

In this approximation, the effective Young's modulus E of the interface is given by

$$E(semi) = \frac{1}{I^2} \frac{k_1}{Q_1} \left(\frac{2A_1(A_{01}^2 - A_1^2)}{R} \right)^{1/2} \quad (5)$$

with

$$\frac{1}{E} = \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_{tip}^2}{E_{tip}} \quad (6)$$

s stands for sample and ν_{tip} , ν_s is the Poisson's ratio of the tip and the sample. Here, we aim to determine the Young's modulus of a layer of thickness h deposited on a rigid material. For this interface, the force exerted by a paraboloid as function of the indentation is determined by the expression deduced by Garcia and Garcia³¹

$$F_{ts} = F_{Hertz} [1 + F_{bec}(a/h)] \quad (7)$$

$$F_{bec} = \left(1.133 \left(\frac{\sqrt{RI}}{h} \right) + 1.497 \left(\frac{\sqrt{RI}}{h} \right)^2 + 1.469 \left(\frac{\sqrt{RI}}{h} \right)^3 + 0.755 \left(\frac{\sqrt{RI}}{h} \right)^4 \right) \quad (8)$$

where a is the contact radius

$$a = \sqrt{RI} \quad (9)$$

By introducing eq. 7 into eq. 1, the expressions for $V_1(E, I)$ and $V_2(E, I)$ are obtained. These equations are detailed in the Supporting information. Those expressions in combination with equations 2 and 3 lead to a systems of equations

$$\begin{cases} V_1(E, I) - V_{1m} = 0 \\ V_2(E, I) - V_{2m} = 0 \end{cases} \quad (10)$$

The solution of the above system provides an analytical and closed-form expression to determine the Young's modulus of a layer with bimodal AFM,

$$E \text{ (layer)} = \frac{\sqrt{8}}{I^2 \sqrt{RA_1}} \frac{V_{1m}}{1 + 1.03 \frac{\sqrt{RI}}{h} + 1.25 \left(\frac{\sqrt{RI}}{h} \right)^2 + 1.14 \left(\frac{\sqrt{RI}}{h} \right)^3 + 0.55 \left(\frac{\sqrt{RI}}{h} \right)^4} \quad (11)$$

We have omitted some intermediate steps⁶⁶ which are detailed in the SI.

Accuracy assessment. To assess the accuracy and modulus range to determine the elastic modulus of ultrathin layers with bimodal AFM, we have performed simulations, experiments and comparisons with force-distance curve measurements.

Simulations. We have used an open-source dynamic AFM code (dForce) to simulate bimodal AFM operation.⁶⁷ Figure 2 shows the dependence of the Young's modulus of the sample (layer) ($E_s = 10$ MPa) as a function of the indentation, thickness of the layer and tip's radius. The figure compares the values obtained by using the expression for a semi-infinite material (eq. 5) with those given by the expression deduced for a finite thickness layer (eq 11). Three different thickness were contemplated, 7 nm, 14 nm and semi-infinite. The first two values were chosen to match the thickness of single and double DCCP lipid bilayers.

The bottom effect correction provides values that coincide or are very close with the nominal value (10 MPa) (Fig. 2a). The semi-infinite expression tends to overestimate the Young's modulus. The overestimation increases with the indentation and could lead to significant errors (several-fold factors). We remark that the stability and accuracy of bimodal AFM measurements

depend on the values of the free and set point amplitudes (1st mode). Therefore, a minimum indentation is needed to probe the elastic response of a material. The value depends on the elastic modulus. For a 10 MPa material, the minimum indentation to get a stable bimodal AFM operation is about 0.5 nm.

Figure 2b shows Young's modulus dependence on the indentation for different tip's radii ($R= 1, 2, 4, 8$ and 10). In bimodal AFM, the maximum indentation is increased by decreasing the amplitude ratio (A_1/A_{01}). The Young's modulus obtained by using the bottom-effect correction (eq 11) shows a region of operational parameters where the Young's modulus might be determined with an error below 10%. This requirement is very hard to achieve if the layer is treated as semi-infinite material (eq. 5). In fact, it only happens for the sharper tip ($R = 1$ nm) and within a very small range of indentations (0.5-0.8 nm).

The results presented in Figure 2 underline a counter-intuitive result in nanomechanical mapping of ultrathin materials. All other factors equal, the error introduced by using a semi-infinite model to determine the elastic modulus increases by increasing the tip radius. This happens because the bottom effect artifact depends on the contact area, which for the same indentation, increases with the tip's radius.

Bimodal AFM nanomechanical maps on lipid bilayers. To illustrate the process of recovering the Young's modulus of an ultrathin soft material, we have performed experiments on lipid layers. This system enables to prepare layers of different thickness (multiples of the bilayer thickness). This feature provides a convenient experimental model to test the validity of the theory. Chiodini *et al.* used lipid bilayers to apply bottom effect corrections to force-distance curves.³⁴ Lipid bilayers are of relevance in cell biology and biotechnology.⁶⁸⁻⁷⁰

Figure 3 shows the topography (Fig. 3a) and Young's modulus maps (Fig. 3b-c) of a region that includes single lipid bilayer and double lipid bilayers domains. Figure 3b shows the elastic

modulus map obtained by using the finite thickness theory (bottom effect correction) while Fig. 3c shows the map obtained by using the semi-infinite theory. The cross-sectional profiles across the lines marked in the maps together with the histograms taken over the whole region are shown in the bottom panels. The height of the single and double bilayers were, respectively, 6 and 12 nm (average values). The Young's modulus determined by using a semi-infinite model depends on the thickness (40 MPa for a single bilayer and 22 MPa for a double bilayer). This no longer the case when the bottom-effect correction is applied. The first bilayer gives a modulus of 11.6 MPa while the 2nd bilayer gives 11.9 MPa. The difference is within the 5 % relative error.

Another interesting observation comes from analysing the dependence of the force on the layer thickness, indentation and tip's radius (Figure 4). For the same indentation, the peak (maximum) force applied on a layer decreases with the layer thickness (Fig. 4a) and increases with the tip's radius (Fig. 4b). Figure 4c shows the experimental values of the indentation (Fig. 4c) and the maximum force (Fig. 4d) exerted on the lipid domains (Fig. 3). For the same experimental conditions, the thinner the layer the larger the force and conversely the smaller the indentation. These curves indicate that influence of the rigidity of the solid support on the force is an intrinsic property of the interface, as such, it is unavoidable. By knowing the layer thickness, the bottom-effect correction manages to recover the correct Young's modulus of the material with independence of its thickness.

To check the consistency of the above value, we have also determined the Young's modulus of the lipid bilayers domains by acquiring force-distance curves. Figure 5 shows the box-plots of the Young's modulus obtained from bimodal (Fig. 5a) and force-volume (Fig. 5b) maps of the same a region of the sample. Again, the use of semi-infinite model and theory provides values which depend on the layer thickness. This artifact is corrected by applying the bottom-effect correction. Significantly, both methods provided similar values for the lipid bilayer with independence of the number of bilayers when bottom effect corrections were applied.

To generalize the results, we have performed simulations for materials ranging from 1 MPa to 100 GPa. Figure 6 shows that the use of a semi-infinite model might lead to a 10-fold overestimation of the Young's modulus of an ultrathin material. On the other hand, the bottom-effect correction recovers the proper Young's modulus with independence of the layer thickness. The simulations illustrate another counter-intuitive feature of the bottom-effect artifact. For ultrathin layers, say below 15 nm, the bottom-effect artifact is also present in a 10 GPa layer. In the bottom-effect correction, the influence of the rigid support is weighted by the a/h ratio.

We remark that the bottom-effect corrections are essential to provide accurate quantitative elastic property values of materials, specifically of ultrathin materials. However, many bimodal AFM applications aim to enhance spatial resolution or material contrast.⁷¹⁻⁷⁸ Those applications do not require any bottom effect correction.

CONCLUSIONS

The force exerted by a tip on an ultrathin material deposited on a rigid support bears the influence of the mechanical response of the support. This is an intrinsic property of the interaction and the structure of the interface. Therefore, to determine a material property of an ultrathin material with bimodal AFM requires to process the instrument observables with a finite thickness model that accounts for the bottom effect artifact.

Bimodal AFM is arguably the most advanced method to determine the nanomechanical properties of materials and interfaces. We have developed a model that enables to recover the intrinsic Young's modulus of a material deposited on a solid support with independence of its thickness by bimodal AFM.

We demonstrate that bimodal AFM provides accurate Young's modulus values of ultrathin layers (1-15 nm). In fact, those values are independent of the layer thickness. The model was validated

for wide range of materials (1 MPa-100 GPa). The validity and accuracy of the bimodal method was tested by simulations, experiments and comparison with force volume measurements.

We remark that the application of conventional contact mechanics models, this is, those which consider the material as semi-infinite leads to overestimate the Young's modulus of an ultrathin material by a factor 10-fold or larger. Contrary to common wisdom, the error in the estimation of the Young's modulus might be significant even for moderate to hard ultrathin materials (10-100 GPa). This might happen whenever the tip-layer contact radius is comparable to the layer thickness.

MATERIALS AND METHODS

Bimodal AFM experiments. The bimodal AFM measurements were performed in the amplitude modulation-frequency modulation (AM-FM) configuration.^{48,64} An introduction to amplitude and frequency modulation modes is provided in ref. 79. The experiments were performed with a Cypher VRS (Asylum Research Inc.). The bimodal AFM observables were processed with a custom-made software. The experiments were conducted in liquid (see below). USC-F0.3-k0.3 microcantilevers (NanoAndMore, Germany) with $f_1=366$ kHz, $k_1 = 0.332$ N m⁻¹, $Q_1 = 1.1$, $f_2 = 1621$ kHz, $k_2 = 6.52$ N m⁻¹ were used to image the lipid bilayer. Proper calibration of the force constants of the excited modes (1st and 2nd)⁸⁰ is essential for accurate nanomechanical mapping. The tips of USC-F0.3-k0.3 cantilevers are made of diamond-like carbon. To deduce the Young's modulus of the lipid bilayers we assumed $\nu_{tip}=0.2$ and $E_{tip}=400$ GPa while the Poisson ratio for the lipid bilayer was 0.3. We have applied the method developed by Labuda *et al.* for calibrating higher eigenmodes.⁸¹ Typical parameters for bimodal AFM experiments on lipid layers were $A_{01} = 2.0$ nm, $A_1 = 1.8$ nm, $A_2 = 0.1$ nm, $F_{max} = 250$ pN, $I_{max} = 1.6$ nm.

Bimodal AFM bottom-effect theory. The intermediate steps to deduce the relationship between the Young's modulus and the bimodal observables are presented in the Supporting Information.

Force-distance curves. Force-distance curves (FDC) on lipid bilayers were obtained with the same cantilever right after performing the bimodal AFM measurements. FDCs were recorded at 2 Hz. The indentation was stopped when the force reached 240 pN. The force-distance curves were fitted with the bottom effect correction deduced for paraboloid tip.³¹

Lipid bilayers. Dipalmitoylphosphatidylcholine (DPPC) powder (Avanti - 850355P-200MG 16:0 PC (DPPC)) was dissolved in Chloroform/Ethanol 2/1 (vol/vol) to a final concentration of 1 mM, evaporated under a nitrogen flow and dried on a desiccator for 2 hours.⁶⁸ The dried lipids were resuspended in buffer (10 mM tris, 100 mM NaCl, 3 mM CaCl₂, pH 7.4) to a final lipid concentration of 1 mM. The solution was sonicated 5 times for 2 minutes while keeping the suspension in an ice bath to obtain the small unilamellar vesicles (SUVs) suspension. 50 µl of the suspension was deposited into a freshly cleaved muscovite mica disk (Grade V-1, Alpha Biotech Ltd.) and incubated for 60 minutes in a closed container to avoid evaporation. To favor the formation of a double bilayer⁶⁹ 50 µl of the DPPC solution was added every 15 minutes. After deposition the sample was carefully rinsed with Imaging buffer (10 mM Tris, 100 mM NaCl, pH 7.4). The sample was placed inside a fluid chamber. AFM imaging started without further delays. The measurements were performed at a temperature of 22 °C.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at DOI:

Additional force volume AFM maps of the lipids bilayers, additional details of simulations and theory.

Figure S1: Force volume maps of single and double lipid bilayers (DPPC).

Figure S2: Numerical simulations of bimodal AFM of a material with different thicknesses

Figure S3: Numerical simulations of bimodal AFM of a finite thick layer for different tip radius

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AUTHOR CONTRIBUTIONS

V.G.G deduced the analytical expressions, and performed the experiments and simulations.

R.G. designed the simulations, the experiments and wrote the manuscript. Both authors discussed the results and revised the manuscript.

Note

The authors declare the following competing financial interest: R.G. is inventor of several patents on bimodal AFM methods.

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FIGURES

Figure 1. Scheme of a tip-ultrathin layer-solid support interface. The layer depicts a single and two lipid bilayers system. The right panel depicts the detection signals in bimodal AFM (AM-FM configuration).

Figure 2. Simulations of bimodal AFM operation for finite thickness and semi-infinite materials. (a) Young's modulus as a function of the indentation for three different sample thickness ($R = 8$ nm). (b) Dependence on the tip's radius for a layer $h = 7$ nm. The solid lines are the results obtained by using the bottom-effect correction (bec). The symbols correspond to the results given by using the semi-infinite model. The stripe (shaded) shows the values that lie within a 10% window from the *true* Young's modulus of the material (10 MPa). The indentation values that produce a stable bimodal AFM operation are marked.

Figure 3. Bimodal AFM topography and elastic modulus maps of a lipid bilayer sample. (a) Topography (top) and height cross-section across the dashed line and height distribution along the map (bottom panels). (b) Young's modulus map obtained by applying a semi-infinite model. (c) Young modulus map obtained by applying a finite thickness model (bottom-effect correction) (top). No differences are observed between the bilayer and the 2-bilayer regions. The bottom panels show the modulus cross-section across the border between a bilayer and a 2-bilayer region and the Young's modulus distribution of the Young's modulus map. The semi-infinite model gives a Young's modulus that depends on the thickness of the number of lipid bilayers.

Figure 4. (a) Maximum force applied in bimodal AFM as a function of the indentation for a 10 MPa material (simulation) $R = 8$ nm. (b) Maximum force applied in bimodal AFM as a function of the indentation for different tips. $E_s = 10$ MPa material; $h = 7$ nm. (c) Experimental values of the indentation obtained from the different regions of the supported lipid bilayers (from figure 3b). (d) Maximum force exerted on the different regions of supported lipid bilayers (from Fig. 3b).

Bimodal	Semi-infinite	Bottom Effect Correction
First Layer	41.1 MPa	12.6 MPa
Second Layer	23.4 MPa	12.9 MPa

Force Volume	Semi-infinite	Bottom Effect Correction
First Layer	47.2 MPa	11.6 MPa
Second Layer	24.6 MPa	11.9 MPa

Figure 5. Comparison between bimodal and force volume. The measurements were performed on a lipid bilayer system with single and double layer domains. (a) Bimodal AFM. (b) Force volume.

Figure 6. Determination of the Young's modulus of an ultrathin material with bimodal AFM (theory and simulations). The simulations involve layers of different thickness and Young's modulus values. Tip radius $R = 2$ nm. (a) The use of a semi-infinite contact mechanics model (Hertz, Sneddon) and bimodal theories based on them, leads to significant overestimations of the Young's modulus (relative error ≥ 100 -1200 %). (b) The bottom-effect correction recovers the *true* Young's modulus of an ultrathin material (1-15 nm) over a 5-orders of magnitude modulus range (1 MPa-100 GPa). The relative error is very small, say below 1%. The fluctuations in the relative error values as a function of the layer thickness ($\leq 0.5\%$) are attributed to errors in the numerical integration. The continuous lines are interpolations for visual aid. Details about the simulations are provided in the SI.