Nanomechanical coupling enables detection and imaging of 5 nm superparamagnetic particles in liquid

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Ultra high resolution, sensitive and minimally invasive characterization techniques are needed to understand hybrid surfaces integrated by organic, inorganic and biological structures in air or liquid¹⁻². Those surfaces might be composed of nanostructures that show small but critical differences in their mechanical, magnetic or electrical properties³⁻⁵. Here we show that a force microscope operated in a bimodal mode enables the simultaneous detection of short and long-range interaction forces. This technique is applied to distinguish protein-caged superparamagnetic particles down to 5 nm. The bimodal method exploits the nanomechanical coupling of the excited modes to enhance the sensitivity of the higher mode to detect changes in material properties. The coupling requires the presence of nonlinear forces. Remarkably, bimodal operation enables to identify changes of slowly varying forces (quasi-linear) in the presence of a stronger nonlinear force. Thus, unambiguous identification of single apoferritin and ferritin molecules in air and liquid is accomplished.

To analyze and understand the performance of complex materials and devices made up of nanostructures of different mechanical, electric and/or magnetic properties requires the development of non-invasive, high resolution and single-pass characterization methods⁶⁻¹². Recently, several multifrequency AFM schemes have been proposed to address the above issue¹³⁻²³. Generically, those schemes exploit the nonlinear character of the tip-surface forces to either activate or detect higher eigenmodes or harmonics and to open new channels to improve imaging and composition sensitivity. In any force microscope (AFM) the spatial resolution is ultimately controlled by the tip size and the length scale of the interaction range while the sensitivity is controlled by the thermal noise of the instrument. However, in practice the resolution and the sensitivity are controlled by two other factors. The use of the lift mode decreases the lateral resolution by a factor comparable to the average tip-separation (lift distance). In addition, the feedback mechanism in the conventional single (monomodal) excitation/detection regime inhibits the use of the phase-shift to detect several interactions. Thus, it limits the resolution and the sensitivity of the instrument when other interactions, for example, non-mechanical forces are present. A central problem in force microscopy is how to improve the spatial resolution and the sensitivity of the instrument at a fixed thermal noise background.

Nanoscale magnetic domains have a central role in several biological and synthetic systems²⁴⁻²⁷. Magnetic nanoparticles are also being used as agents for cancer treatment^{1,24} or can be used to stimulate different cell functions²⁵⁻²⁶. Similarly, very small magnetic nanoparticles with a high anisotropy could be used for high-density data storage media²⁸. Those applications require the simultaneous imaging, detection and eventually separation of forces of different nature, for example mechanical and magnetic. Imaging of magnetic structures and nanoparticles with sub-50 nm spatial resolution in air is usually accomplished with a magnetic force microscope^{12,27,29-30} (MFM). This technique works in a two-pass mode. In the first pass, the topography is detected while in the second pass the tip is retracted several nanometers from the imaging mode and the magnetic force is detected (lift mode). The fact that in MFM the tip is placed several nanometers above the surface (lift distance) implies that detecting and imaging magnetic or polarized superparamagnetic particles by magnetostatic interactions is not possible below 10-12 nm in size^{12,29-30}. Furthermore, MFM imaging of nanoparticles does not always separate electrostatic from magnetic interactions³².

Here we develop bimodal force microscopy to simultaneously detect short and long-range forces of different nature. The method is applied to image magnetic polarized single ferritin molecules in air and liquid with a 5 nm spatial resolution. Ferritin is a cage-shaped biomolecule that accommodates an iron oxyhydroxide nanoparticle³³. This protein is formed by a polypeptidic hollow shell (apoferritin) that encapsulates an iron-based core that can reach a maximum diameter of 7 nm. The structure of the iron core resembles that of ferrihydrite with a nominal formula $(\text{FeOOH})_8(\text{FeOH}_2\text{PO}_4)$.

Figure 1 shows a scheme of the bimodal AFM experiments performed in liquid on a surface containing ferritin molecules. The light blue half-sphere represents the liquid cell (Fig. 1a). Bimodal AFM involves the mechanical excitation of two cantilever eigenmodes. As a consequence of the multifrequency excitation of the cantilever, the deflection (photodiode signal) contains two dominant frequencies

$z(t) = A_1 \cos(\omega_1 t - \phi_1) + A_2 \cos(\omega_2 t - \phi_2) + O(\varepsilon)$. The amplitude of the low frequency eigenmode A_1 is used to track the topography of the system (Fig. 1b) while the phase shift of the high frequency component ϕ_2 contains contributions from mechanical and magnetic interactions that allow us to reconstruct the structure of the protein (Fig. 1c). The deflection might have other high order terms $O(\varepsilon)$, however, those components are usually very small and can be neglected. Bimodal atomic force microscopy has demonstrated the ability to resolve the structure of antibodies (IgM) in air and liquid²¹. This method is also compatible with the presence of mechanical¹³⁻¹⁴, electrostatic²² or magnetic interactions²³.

To illustrate the ability of the technique to separate nanostructures of identical short-range mechanical properties but with different long-range interactions, we have imaged surfaces covered with a mixture of ferritin and apoferritin molecules (Fig. 2a). The measurements have been obtained under the presence of a magnetic field of 80 mT to polarize the ferritin superparamagnetic cores. The bimodal phase shift ϕ_2 cross-section of the molecules marked in Fig. 2b shows significant differences (Fig. 2c). Those differences are attributed to the different type of tip-molecule interactions either mechanical (apoferritin) or mechanical and magnetic (ferritin). We remark that the topography of the molecules marked in Fig. 2b is very similar.

The histogram obtained from the bimodal phase image can be separated in three regions (Fig. 2d), $\phi_2 \in [0.1^\circ, 0.5^\circ]$, $\phi_2 \in [0.7^\circ, 1.1^\circ]$ and $\phi_2 \in [1.1^\circ, 1.4^\circ]$. The region with $\phi_2 < 0.5^\circ$ concentrates 80% of the measurements at $0.3^\circ \pm 0.1^\circ$. Interestingly, those nanoparticles also show height values between 2 and 4 nm. We attribute those structures to apoferritin shells. In the intermediate region the nanoparticles are concentrated at $\phi_2 = 0.9^\circ \pm 0.1^\circ$. In this region the observed value of ϕ_2 increases with the height of the nanoparticle. Finally, the nanoparticles showing the largest phase shifts have an apparent height of 7 nm. The correlation observed between the phase values and height measurements is attributed to the nanoparticles made up of an apoferritin shell and an iron oxide core. In fact, we propose that the correlation observed between phase shift and height values in the intermediate region can be explained by the size of the iron oxide core. In the case of a phase shift of 0.9° we estimate a partially filled ferritin molecule with an iron core of 5 nm in diameter. Controlled experiments performed with homogeneous samples made up of either apoferritin or ferritin molecules confirm the above conclusions. The genuine magnetic contrast of the ferritin molecules has been verified by showing that the bimodal phase shift changes with the strength of the magnetic field (Supplementary information, Fig. S1). Furthermore, the sign of the phase shift is reversed by inverting the polarization of the AFM probe (Figs. S2 and S3).

In Fig. 3a we show two images obtained in buffer (pH=3) of a mixture of ferritin and apoferritin molecules. The topography shows a random distribution of nanoparticles. On the other hand, the bimodal phase shift image (ϕ_2) shows two different molecules, ring-like and flat disk molecules (Figs. 3b, c). The ring-like structure is given by ferritin while the flat-disk is given by apoferritin molecules. The phase shift cross-section of the molecules marked in Fig. 3b shows that an apoferritin molecule is characterized by a rectangular shape while a ferritin molecule is characterized by the presence of two peaks and a dip in between. This observation is in stark contrast with the bimodal AFM images obtained in air where apoferritin and ferritin showed a similar phase shift shape but the ferritin gave a stronger signal (Fig. 2c). The ring-like shape of ferritin in liquid is explained by the interplay between magnetic and mechanical interactions and the enhancement of magnetostatic interactions in the bimodal phase shift. Dynamic AFM imaging in liquid is performed under a net repulsive force. When the tip is over an apoferritin molecule, the short-range mechanical forces give rise to the topography and the bimodal phase shift images. When the tip is over a ferritin molecule, the short-range mechanical forces control the amplitude decrease as in the apoferritin case and the topography is recorded. However, the bimodal phase shift is sensitive to the presence of both short-range mechanical (repulsive) and long-range magnetic (attractive) forces. Those forces drive the bimodal phase shift in opposite directions (Fig. 3d). When the tip is on top of the ferritin the magnetic interaction is maximized and a depression in the phase shift-cross section is observed. Furthermore, to validate those conclusions we have imaged a surface composed of gold (hard), ferritin (medium) and apoferritin (soft) nanoparticles (Figs. S4 and S5). Only the ferritin molecules gave rise to ring-like structures, so the presence of a hard inorganic core is not a relevant factor in the reported contrast.

We perform a two-step theoretical study to understand the interplay among eigenmodes, mechanical and magnetic forces that give rise to the enhancement in sensitivity observed in bimodal operation. First, we discuss some analytical expressions that relate the

phase shift to the gradient of the net tip-surface force. Then, we discuss the origin of the enhanced magnetic sensitivity in bimodal AFM in terms of the expressions deduced from a weakly perturbed harmonic oscillator,

$$\phi(\omega_i) = \arctan\left(\frac{\gamma\omega_i}{k_{eff} - m\omega_i^2}\right) = -\arctan\left(\frac{k_i/Q_i}{F'}\right) \quad (1)$$

with

$$k_{eff} = k_i - F' = k_i - \frac{dF}{dz} \quad (2)$$

and

$$\gamma\omega_i = \frac{m\omega_i\omega_i}{Q_i} = \frac{k_i}{Q_i} \quad (3)$$

where k_i , ω_i and Q_i are, respectively, the force constant, resonant frequency and quality factor of the eigenmode i .

By assuming that the force changes smoothly when the tip moves from two neighbor positions 1 and 2 of the molecule, the associated phase shift change is given by

$$\Delta\phi = \phi(2) - \phi(1) = -\frac{1}{1 + \left(\frac{k_i/Q_i}{F'}\right)^2} \frac{k_i}{Q_i} \frac{-\Delta F'}{F'^2} = \frac{k_i/Q_i}{F'^2 + (k_i/Q_i)^2} \Delta F' \approx \frac{Q_i}{k_i} \Delta F' \quad (4)$$

where $\Delta F' = F'(2) - F'(1)$. Thus, changes in the phase shift reflect changes in the differences of the gradient of the forces. Now, for a rectangular cantilever,

$$\frac{Q_i}{k_i} = \frac{\omega_1}{\omega_i} \frac{Q_1}{k_1} \quad (5)$$

as a consequence under monomodal excitation/detection the compositional contrast decreases by using higher order cantilever modes. Numerical simulations confirm the above result in the presence of slowly varying forces (quasi-linear region, Fig. 4a). However, the

bimodal excitation couples the cantilever modes in the presence of nonlinear tip-surface forces. Given the difference in amplitudes ($A_1 \approx 12A_2$), the coupling modifies primarily the properties of the second mode. Thus, the phase shift of the second mode is able to detect changes in both the force and the gradient of the tip-surface forces when the tip moves from one region to the other (Fig. 4b). This is the source of contrast observed in the ferritin images. On the other hand, any compositional contrast in the phase-shift of the first mode is suppressed by the feedback which is controlled by the value of the amplitude of the first mode³⁴ (Fig. 4b).

The phase-amplitude maps of a ferritin molecule deposited on a mica surface (air) for the first and second mode are shown, respectively, in Figs. 4c and 4d. For both modes, the measured phase shift is very close to the conservative trajectory (continuous line). This indicates the conservative and non-destructive character of the tip-surface interaction. A quantitative analysis of the ϕ_1 measurements obtained from the phase shift maps shows that the measurements obtained on a ferritin molecule overlap with those obtained on a mica surface (Fig. 4e). This confirms that the first mode carries no contrast between ferritin and mica. On the other hand, the histogram obtained from ϕ_2 reveals two spots (Fig. 4f). The left spot corresponds to the value obtained on the mica and the right one corresponds to the interaction between the tip and the ferritin molecule.

In conclusion, we report a nanomechanical effect that enables the simultaneous recognition of short-range and long-range forces. The nanomechanical coupling between the first two cantilever modes is established by the nonlinear character of the probe-surface forces. A remarkable property of the coupling is the enhancement of slowly varying forces on the region where another and stronger force is present. This effect has enabled the simultaneous imaging of mechanical and magnetic interactions in a ferritin molecule. In addition, we have demonstrated the magnetic imaging of nanoparticles with an estimated size of 5 nm.

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Author contributions

C.D. , E.T.H. and R.G conceived and designed the experiments. C.D. and E.T.H performed the experiments. J.R. Lozano and E.T.H performed the theoretical calculations. R.G. directed the research and wrote the manuscript. All the authors discussed the results and revised the manuscript.

Methods

The bimodal excitation/detection was performed by using a custom-built electronic unit. This unit was coupled to a commercial AFM base (Multimode AFM, Veeco Instruments Inc., Santa Barbara, USA). The first two fundamental eigenmodes of the cantilever were excited simultaneously by the bimodal unit. We have used supersharp cantilevers (SSS-MFMR NanoAndMore GmbH, Wetzlar, Germany) coated with a thin layer of cobalt (≈ 25 nm). The frequencies for the first and second eigenmode of the cantilevers in air were, respectively, in the ranges of 55 – 65 kHz and 350 – 410 kHz; the nominal value of spring constant for the fundamental mode was $k_1 = 1.0 - 1.4$ N/m and the quality factor in air $Q_1 \approx 270$. The cantilever was excited to free amplitudes of $A_{01} \approx 10$ nm for the first eigenmode and $A_{02} \approx 0.5 - 1.0$ nm for second eigenmode (air) and $A_{01} \approx 7$ nm and $A_{02} \approx 1 - 2$ nm (liquid). The imaging a ratio between the amplitude setpoint and the free amplitude of the first eigenmode (A/A_{01}) was about 0.9. The instrumental error in the phase shift is about 0.01° . More details about the sample preparation and the imaging processing are found in the accompanying Supplementary information file.

To describe the dynamics of the cantilever-tip system we model the cantilever as a rectangular beam. The Euler-Bernoulli partial differential equation of the AFM is approached by a system of n second order differential equations, one for each eigenmode of the cantilever. We also assume that the dynamics of the system is mostly contained in the first two eigenmodes.

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Figure Captions

Figure 1. Scheme of bimodal AFM operation to measure simultaneously mechanical and magnetic interactions. **a**, The first two cantilever-tip eigenmodes (ω_1, ω_2) are excited by applying a mechanical force at the cantilever base. Ferritin molecules are imaged in liquid under the presence of an external magnetic field. **b**, The amplitude of the first mode is used to generate an image of the topography in exactly the same way as in amplitude modulation AFM. **c**, The phase shift of the second mode ϕ_2 is used to reconstruct the structure of the protein with its mechanical (protein shell) and magnetic (iron oxide core) contributions (Supplementary information).

Figure 2. Topography and bimodal phase shift images in air of a deposition containing ferritin and apoferritin molecules. **a**, Topography ($A_{01}=10$ nm, $A_{sp}=9$ nm, $A_{02}=A_2=0.9$ nm). **b**, Bimodal phase shift image of (a). **c**, Phase shift cross-section of the nanoparticles marked in (a) and (b). **d**, Histogram of phase shift ϕ_2 (black) and mean height values (grey) of the nanoparticles. The data has been obtained from several images as the one shown in Figs. 2a and b. Phase shift measurements range from 0.1° to 1.4° . Three main regions can be defined, $\phi_2 \in [0.1^\circ, 0.5^\circ]$, $\phi_2 \in [0.7^\circ, 1.0^\circ]$ and $\phi_2 \in [1.1^\circ, 1.4^\circ]$. Those regions are associated with three different type of nanoparticles, apoferritin shells, partially filled ferritin cores and saturated ferritin. The images have been acquired under the presence of a magnetic field of 80 mT. Scale bar is 50 nm.

Figure 3. Topography and bimodal phase shift images in buffer (pH=3) of a deposition containing of ferritin and apoferritin molecules. **a**, Topography ($A_{01}=7$ nm, $A_{sp}=6$ nm, $A_{02}=1.1$ nm). **b**, Bimodal phase shift image of (a). The phase image shows two different structures are observed, ring-like and full nanoparticles. **c**, Phase shift cross-section of the nanoparticles marked in (a) and (b). **d**, Histogram of phase shift ϕ_2 (black) and height values (grey) of the iron oxide core and apoferritin shell of the nanoparticles. The phase shift of the iron oxide core has been obtained by subtracting the phase shift given $A_1=6$ nm by the mechanical forces of the protein shell and the one obtained in a ferritin nanoparticle (ring-like structure). The data has been obtained from several images as the one shown in Figs. 3a and b. Scale bar is 100 nm.

Figure 4. Phase-amplitude maps in bimodal AFM. **a**, Dependence of the phase-shift of eigenmodes 1 and 2 on the tip-surface distance under bimodal excitation. The phase shift of the lowest mode is more sensitive to changes on the tip-surface force in the quasi-linear region because it has a higher Q/k ratio. However, the presence of nonlinear forces enhances the sensitivity of the second mode to provide material contrast. **b**, Phase-shift dependence on the magnetic field in bimodal AFM. Only ϕ_2 carries information on the properties of the interaction. **c-d**, Phase shift map as a function of the setpoint amplitude for ferritin on mica. **e**, Histogram from **c** of phase shift values on mica and ferritin (first mode). **f**, Histogram from **d** of phase-shift values on mica and ferritin (second mode).

Figure 1

Figure 2

Figure 3

Figure 4