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Dependence of the volume of an antibody on the force applied in a force microscopy experiment in liquid

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ABSTRACT

The volume of a protein can be estimated from its molecular weight. This approach has also been applied in force microscopy experiments. Two factors contribute to the determination of the volume from a force microscope image, the applied force and the tip radius. Those factors act in opposite directions. Here, we demonstrate that in the optimum conditions to image a protein, the apparent volume deduced from an AFM image overestimates the real protein volume. The lateral broadening due to the tip finite size, makes the simulated volume to exceed the real protein volume value, while the force applied by the tip tends to decrease the measured volume. The measured volume could coincide with the real volume for either a point-size tip at zero force or when the compression exerted by the tip compensates its dilation effects. The interplay between the above factors make unsuitable to apply the molecular weight method to determine the volume of a protein from AFM data.

1. Introduction

The atomic force microscopy (AFM) has shown the capability to reveal high resolution images of a variety of protein membranes in liquid [1–3]. It has also resolved the pitch of double stranded DNA [4,5] and RNA molecules [6]. Images of isolated antibodies have resolved the different domains of the protein [7]. It is known that the finite size of the probe introduce a tips dilation effect that increases the biomolecule size [8]. The geometry of biomolecules provided by the AFM could also be distorted by the force applied on them [9,10], by differences in the interaction forces between the biomolecules and the surrounding substrate [11] or by pH and ionic concentration [12]. Nonetheless, the volume extracted from AFM images have been correlated to the expected protein's volume as a function of its molecular weight [13–16], by applying the method proposed by Cantor and Schimmel in 1980 [17] (volume based on the molecular weight hereafter).

By characterizing different proteins with molecular weights ranging from 38 to 900 kDa with the AFM, Schneider et al. [14] reported a linear correlation between the molecular weight and the volume measured by AFM. This result implied that it was possible to infer the molecular weight of an unknown protein, single or multimeric, according to its measured volume. More interestingly, the measured volume coincided with the predicted one, according to the molecular weight of the proteins. Experimental conditions like the tip radius or the environment (air or liquid) did not seem to affect their results. The proteins volume was calculated as a spherical cap (see Section 2). They used the full width at half maximum (FWHM) to compensate the size overestimation due to the tip-sample convolution.

Measurements performed by Neaves et al. [15] confirmed the linear relationship between the measured volume and the molecular weight. However, those measurements provides smaller volume values. The measured volume was approximately 1/3 of the value reported by other authors (including Schneider et al. [14]). They concluded that the discrepancy was related to their AFM setup. They used more sharpen cantilevers with higher spring constant which, in one hand, decrease the lateral broadening and, additionally, exert higher force on the sample. For that reason, they suggested to use the basal width instead of the FWHM in the spherical cap model (see Section 2). With that correction, they found a better agreement between the measured volume and the expected value, according to the molecular weight of the proteins.

Recent experiments performed by Fuentes-Perez et al. [16] confirmed the linear correlation between the molecular weight and the volume, and extended it to single-stranded DNA (ss-DNA) and double-stranded DNA (dsDNA). In their work, they co-adsorbed DNA and proteins. The volume of the DNA was used as reference to normalize the volume of the proteins. As the spherical cap model does not apply for a fiber like DNA, they used a direct

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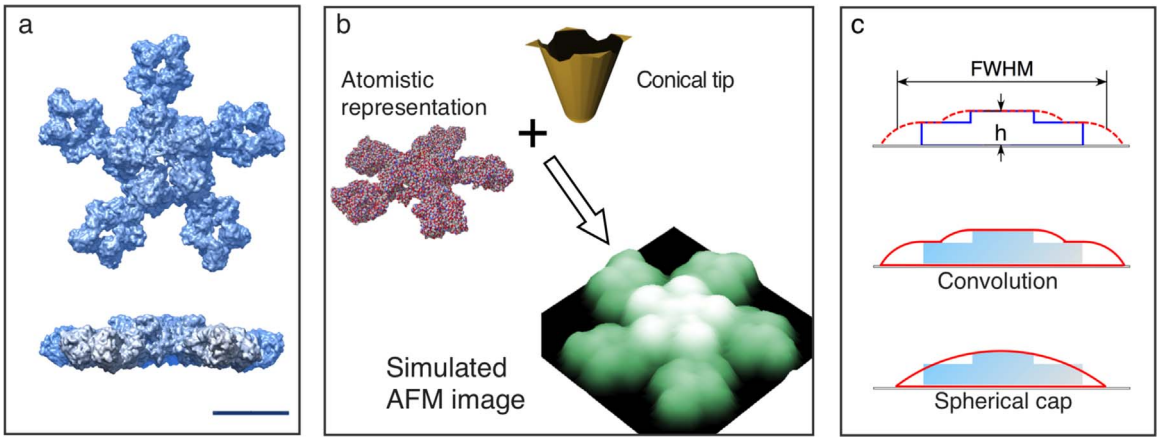

Fig. 1. (a) Top and lateral view of the IgM antibody. Scale bar, 10 nm. (b) Simulated AFM images are based on the convolution between the IgM and a conical tip. (c) Schemes of tip dilation effects for a direct model and a spherical cap model. Relevant dimensions indicated are the full width at half maximum (FWHM) and the height (h). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

method based on the tip-sample convolution to estimate the volume of the biomolecules. In their work, the volume estimated as the tip sample convolution coincided with the expected value deduced from the molecular weight of the proteins.

Imaging proteins with high-resolution requires using sharp tips and applying low forces. Amplitude modulation AFM (AM-AFM) is widely used to study isolated proteins since the applied lateral forces are lower than in contact mode. In 2000, San Paulo and Garcia [9] resolved the two Fab and the Fc fragments of immunoglobulin-G (IgG) antibodies. This was a significant step in high resolution imaging of isolated proteins in air because the IgG antibodies were previously observed as single globular structures. Nowadays, it is possible to achieve high-resolution images of IgG antibodies routinely, even with non-supersharp tips by controlling the experimental conditions [18–20]. Another key factor when calculating the apparent volume of proteins is the apparent height loss. The measured height of the antibodies in air from high resolution AFM images is usually smaller than the nominal value by a factor 2. Santos et al. [11] demonstrated that the nanoscale dimensions of the sample induce an apparent height loss in ambient conditions. This fact will also affect the measured volume.

Regarding IgM antibodies, Czajkowsky and Shao [21] proposed a model that predicted a non-planar, mushroom-shaped complex for the molecule (i.e., the central portion formed by the C-terminal domains protruding out of the plane formed by the Fab domains); it was confirmed by cryo-AFM of individual molecules. That shape was confirmed in liquid too by Martinez-Martin et al. [7], who measured a height in the protrusion around 6 nm, by using bimodal frequency modulation AFM. The force, estimated in 30 pN, was reconstructed by applying the method proposed by Sader and Jarvis [22] in 2004.

A hybrid dynamic AFM mode has been applied to determine the stress-strain curve of a single IgM pentamer in liquid [10]. This method enables the application of forces from 20 to 200 pN. Even at those very small forces, the protein height is 6 nm, this is, 1 nm smaller than its nominal height. This data shows that protein deformation could be unavoidable for some biomolecules, such as antibodies, that have an average Young modulus of 10 MPa.

Here, we analyze the influence of the applied force and the tip radius to determine the volume of IgM antibodies from AFM images. We combine experiments and image reconstruction simulations. First, we use the structure of the IgM antibodies proposed by Czajkowsky and Shao [21] to simulate the AFM images of IgM for different tip radii. Then, we determine the volume from those images. Second, we acquire images of IgM antibodies by applying different forces. From those images we determine the experimental volume. The results are compared with the theoretical volume based on the molecular weight.

2. Materials and methods

2.1. Theoretical volume based on the molecular weight

The IgM volume based on its molecular weight was estimated by the equation proposed by Cantor and Schimmel [17] and described elsewhere [13–16]:

$$V = \frac{M_w}{N_A}\left(\bar{V}_2 + \delta\bar{V}_1\right) \quad (1)$$

where V is the protein's volume; M_w is the known molecular weight of the protein; N_A is the Avogadro's number; $\bar{V}_2$ and $\bar{V}_1$ are the partial specific volumes of protein (0.74 cm^3/g) and water (1 cm^3/g), respectively; δ is the extent of protein hydration (0.4 g H_2O/g protein).

The extent of protein hydration, estimated by calorimetric methods, does not include bulk water trapped in cavities and holes, which does not bind tightly but will contribute to the protein's apparent size. In addition to that, the complexities due to the ionic strength are ignored, so the model considers a two-component system of water (component 1) and the macromolecule (component 2) [17].

The molecular weight of the IgM was calculated as the sum of each amino acid of the protein's sequence. For the IgM (code 2RCJ of the Protein Data Bank (PDB) [23]), we got a molecular weight M_w=851.97 kDa. According to Eq. (1), the protein's volume is 1613 nm^3.

2.2. Simulation of the AFM images of antibodies

Fig. 1a shows the top view and lateral views of the IgM antibody model [21] that has been used here to simulate the AFM images. The atomistic representation of the IgM was generated by using the Chimera package [24]. The model was represented in a gray scale and depth cued to associate the color with the height of the atoms in the top view. To simulate the effect of the tip size on the image provided by AFM we have used the following approach. Tagged image file format (TIFF) files of the atomic model of IgM were processed by using the tip dilation option in the Gwyddion software [25] that enables to generate AFM-like images as a function of the tip radius. The height of the IgM is an input. Here

we have used a height of 7 nm for the top central region [10]. The tip was simulated as a cone with opening angle of 35°. The cone ended in a spherical tip. Tips with radius in the 0.15–8 nm range have been used. The tip dilation algorithm assumes that the tip and the molecule are non-deformable bodies (Fig. 1b).

2.3. Sample preparation

Human IgM antibodies were purchased from Chemicon, Inc., USA. First, a 20 μ l drop of $NiCl_2$ (50 mM) was deposited on a freshly cleaved mica surface for 30 s. The $NiCl_2$ is deposited to functionalize the mica surface to enhance the adsorption of the antibodies. Then a 5 μl drop taken from a 40 μg/ml of IgM antibodies diluted in phosphate buffered solution (PBS) was injected into the $NiCl_2$ drop. After an incubation period of 60 s, the sample was rinsed with milliQ water in order to remove the proteins that were weakly attached to the surface.

2.4. AFM imaging

The experiments were performed in liquid with a Cypher S microscope (Asylum Research, Santa Barbara, USA). The images were acquired by using the hybrid dynamic AFM [10,26] with AC-40TS (Olympus) cantilevers, characterized by spring constant $k_c \approx 0.07$ N/m, resonant frequency $f_0 = 25$ kHz, tip radius $R = 8$ nm and quality factor in liquid $Q \approx 2$. In this mode, the driving force and the oscillation amplitude are fixed. The frequency of the oscillation is tuned to the actual resonant frequency during the imaging process. The hybrid dynamic AFM mode has two main feedback loops. The first loop keeps the amplitude of the oscillation at a fixed value. The second loop tracks the actual resonant frequency of the microcantilever so the oscillation is always phase shifted 90°with respect to the driving force. The amplitude of the force that drives the oscillation of the cantilever V_{exc} is used as the topography feedback. This mode enables a robust imaging process in air and liquid.

2.5. Force reconstruction

To transform the observables comprised in an AFM image into a quantitative value of force, a force curve was recorded on top of an IgM antibody after every measurement. Then we used the Sader-Jarvis force reconstruction algorithm [22] to obtain the applied force:

$$F_{ts}(d) = 2k\int_d^\infty X_{SJ}dx + 2k\int_d^\infty \frac{\sqrt{A}}{8\sqrt{\pi(x-d)}}X_{SJ}dx - 2k\int_d^\infty \left(\frac{A^{3/2}}{\sqrt{2(x-d)}}\frac{\partial X_{SJ}}{\partial d}\right)dx \quad (2)$$

$$X_{SJ} = \frac{\Delta f(d)}{f_0} \quad (3)$$

where k is the spring constant of the cantilever, F_{ts} is the interaction force between tip and sample, f_0 is the unperturbed resonant frequency, Δf is the change in the resonant frequency, A is the amplitude of oscillation and d is the distance of closest approach between tip and sample during an oscillation cycle.

2.6. Volume calculation methods

We have used two methods to estimate the volume of the antibody. The first method is the so called direct method. In this method the volume is calculated from the pixels in the AFM image within a region of interest [16]:

$$V = V_{total} - V_{bkg} \quad (4)$$

where V_{total} is the total volume of the particle based on its pixels' height, including the substrate, and V_{total} is the fraction of the total volume that corresponds to the background (Fig. 1c, middle).

The second method is a geometric method. The volume is calculated by considering the protein as a spherical cap (Fig. 1c, bottom):

$$V = \frac{\pi h}{6}\left(3\left(\frac{W}{2}\right) + h^2\right) \quad (5)$$

where h and W are, respectively, the height and width of the protein. In our case, we considered W as the full width at half maximum (FWHM) [14,27]. For the experimental data, topography images recorded at forces in the range 22–240 pN were used to estimate the IgM volume. The images were flattened and analyzed with Gwyddion. Typically, 12 antibodies were processed from each AFM image to report the mean and standard deviation on the volume.

3. Results and discussion

3.1. Influence of the tip radius and the lateral broadening on the measured volume

Fig. 2a displays 6 AFM images (simulations) obtained by using the IgM structure and tip dilation features described above. The tip radius has been changed from 0.15 nm, approximately the radius of a water molecule, to 15 nm. As expected, the lateral broadening of the simulated AFM images increases by increasing the tip radii. Only for R values below 1 nm, the images resolve the interspacing between the Fab domains of a given monomer. The overall pentameric structure of the antibody is retained up to a $R = 8$ nm. For R values above 15 nm, the AFM image gives a globular, featureless image of the protein. From the above images we can determine the volume (direct method) as a function of R (Fig. 2b). The plot shows that even for a tip as small as a water molecule ($R = 0.15$ nm), the measured volume is larger than the value provided by Eq. (1). The simulated volume exceeds the theoretical volume in 900 nm^3 (there is an overestimation of the volume of 55%). If we move to the other side of the graph, for $R = 8$ nm, the difference is considerably larger: the spherical cap method will overestimate the volume in 2170 nm^3 (134%) and the total convolution method in 4690 nm^3 (290%).

The volume also increases with R when estimated by using the spherical cap method. This method provides values closer to the value obtained by Eq. (1). For $R = 0.15$ nm it matches the theoretical volume. It seems that the spherical cap approximation compensates the lateral broadening introduced by the tip. In all other cases, the measured volume is larger than the theoretical value determined from Eq. (1).

The above results have been obtained by assuming that the antibody is a rigid and non-deformable object. The effective Young modulus of an IgM has been measured and it gives values in the range 2.5–9 MPa [10]. Those values indicates that the antibody will compress under the action of very smaller forces. The forces apply in AFM during imaging will deform the protein and, consequently, the measured volume will be smaller than the ones predicted in the simulations.

3.2. Influence of the applied force on the measured volume

To determine the influence of the applied force on the

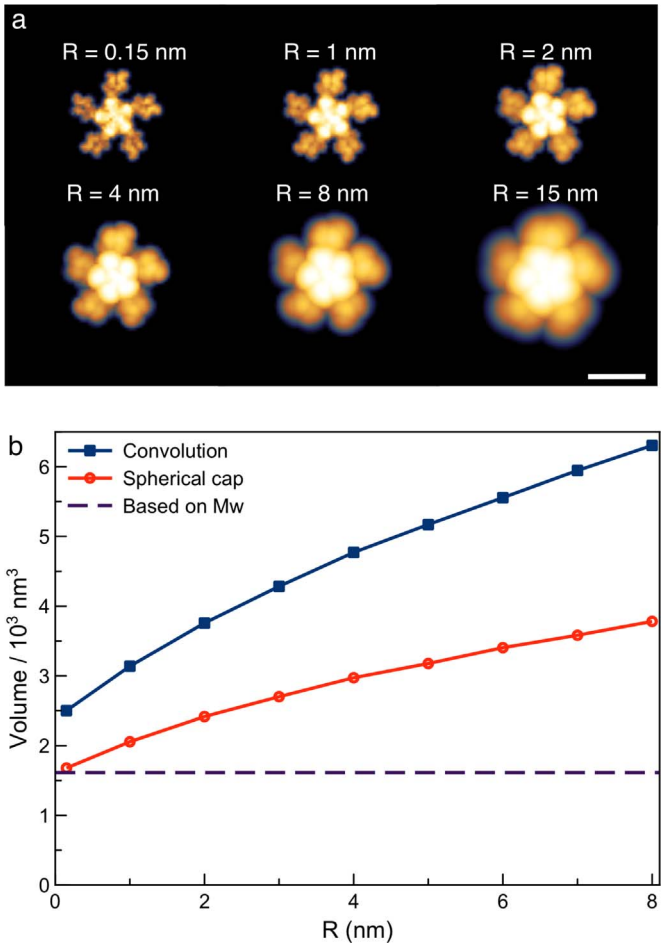

Fig. 2. Influence of the tip radius (R) on the measured volume. (a) Simulated images of the tip-IgM convolution. Scale bar, 20 nm. Color scale, 7 nm. (b) Calculated volume of the simulated IgM antibodies for R=0.15–8 nm. The volume was estimated with the direct method (Eq. (4)) and the spherical cap model (Eq. (5)). The volume based on the molecular weight (Eq. (1), dashed line) was plotted as reference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

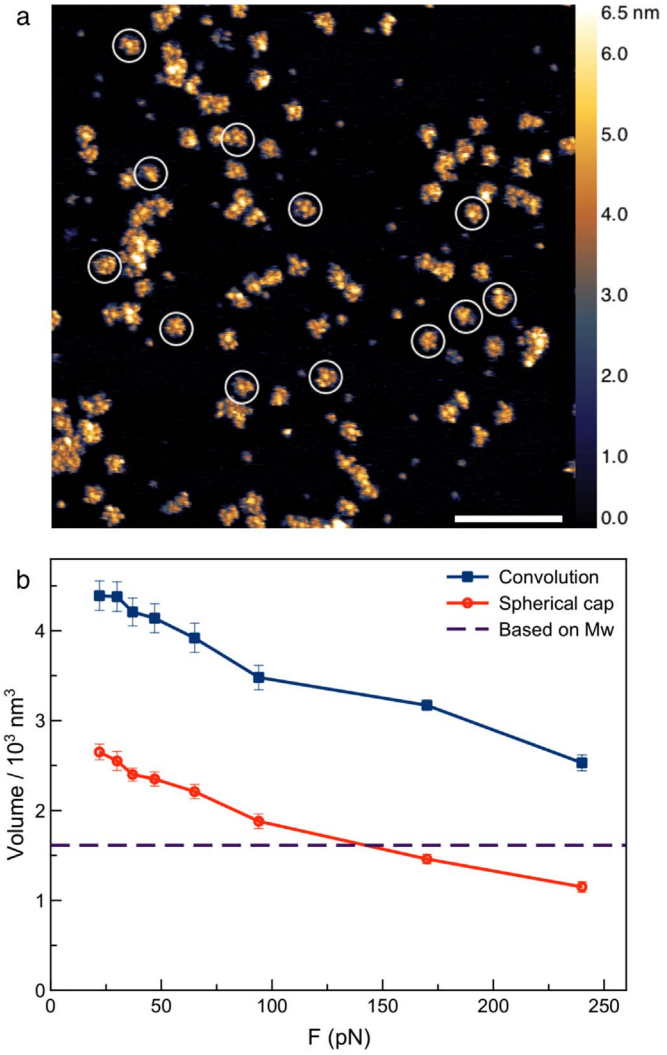

Fig. 3. Influence of the applied force on the measured volume. (a) AFM topography image of IgM antibodies on mica. Experiments performed in water; applied force, 30 pN. Scale bar, 200 nm. (b) Isolated antibodies (like those circled in (a)) were used to get the mean volume. Error bars represents the standard deviation. The volume was estimated as the tip-sample convolution (Eq. (4)) and the spherical cap model (Eq. (5)). The volume based on the molecular weight (Eq. (1), dashed line) was plotted as reference.

measured volume, IgM antibodies were measured under forces ranging from 22 to 240 pN. IgMs are distributed in the surface in random positions, sometimes grouped or isolated, and some of them are fragmented as can be observed in Fig. 3a. For the volume study, only isolated IgMs with a recognizable pentameric structure have been considered..

Fig. 3b shows the dependence of the volume for the direct and spherical cap methods as a function of the applied force. Each data point represents the average over 12 IgMs. We have not applied forces higher than 240 pN because they introduce plastic deformations on the antibodies [10]. The horizontal line is theoretical volume obtained from the molecular weight of the IgM (Eq. (1)). For both models, the volume decreases by increasing the applied force. We observe that, at the smallest force applied here (22 pN), the measured volume exceeds the theoretical volume in 1040 nm^3 (64%) and 2780 nm^3 (172%), respectively, for the spherical cap and the direct methods. This result illustrates that the lateral broadening introduced by the tip contributes to the measured volume.

In the range of 100–175 pN forces, the spherical cap method gives an apparent volume close to the theoretical volume of the protein. This is the only range of applied forces in which the measured volume coincides with the theoretical one. This coincidence, however, should be considered an artifact. It comes by the compensation of two factors, the compression of the antibody by the applied force and tip dilation effect.

The consistency of above results can be checked by deducing the tip radius by comparing the volume at zero force extrapolated from the AFM data (Fig. 3b) with the volume predicted by the simulations for a certain tip radius. We observe that the volume at zero force (extrapolation) is $V(0\ pN)=3000\ nm^3$ for the spherical cap and $V(0\ pN)=4750\ nm^3$ for the direct method. These values can be correlated with the simulated data in Fig. 2b. Both methods provide a radius of 4 nm.

4. Conclusions

We have studied the influence of the tip radius and the applied force on the volume of an IgM antibody obtained from force microscopy. We have also studied the validity of using volumetric methods to determine the volume of proteins from AFM images. To determine the dependence of the applied force on the measured volume, we have taken images of IgM antibodies in liquid for forces ranged in 22 pN to 240 pN range. We show that the volume decreases by increasing the applied force. This is due to

the compression of the biomolecule. On the other hand, simulations show that the apparent volume of a protein increases by increasing the tip radius. The volume measured in an AFM experiment will always be greater than the real volume of the protein. The effect of the applied force and the tip radius could be such that the AFM image provides a volume that coincides with the real value. This could happen for forces in the 100–200 pN and radius in the 4–7 nm range. However, this coincidence should be considered fortuitous.

Finally, if volumetric analysis is used to infer the molecular weight or the multimerization state of proteins, it is highly recommended to control that the experimental conditions (i.e., applied force and tip radius) are the same.

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