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# An overview of the biophysical applications of atomic force microscopy

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**Abstract**

The potentialities of the atomic force microscopy (AFM) make it a tool of undeniable value for the study of biologically relevant samples. AFM is progressively becoming a usual benchtop technique. In average, more than one paper is published every day on AFM biological applications. This figure overcomes materials science applications, showing that 17 years after its invention, AFM has completely crossed the limits of its traditional areas of application. Its potential to image the structure of biomolecules or bio-surfaces with molecular or even sub-molecular resolution, study samples under physiological conditions (which allows to follow in situ the real time dynamics of some biological events), measure local chemical, physical and mechanical properties of a sample and manipulate single molecules should be emphasized.


*Keywords:* Atomic force microscopy; Nucleic acid; Supported membrane; Protein; Polysaccharide; Non-topographical application

## 1. Introduction

The atomic force microscopy (AFM) or scanning force microscopy (SFM) is included in a wider group of techniques named scanning probe microscopies (SPM). In all these techniques, the surface of the sample is scanned by a probe, following parallel lines, measuring a local interaction in the near-field region, and registering its value for each position (Fig. 1). Thus, the probe is always the basic component of SPM, conditioning the resolution of each microscope. All the techniques of this family evolved from the invention of the scanning tunneling microscopy (STM) in 1982 [1]. Four years later, through the adaptation of one of these microscopes, Binnig, Quate and Gerber created the first atomic force microscope [2].

It should be stressed that AFM and STM are only the most used and further developed SPM techniques. While AFM is based on the detection of repulsive and attractive surface forces, and STM on the electron tunnel effect, there are other SPM

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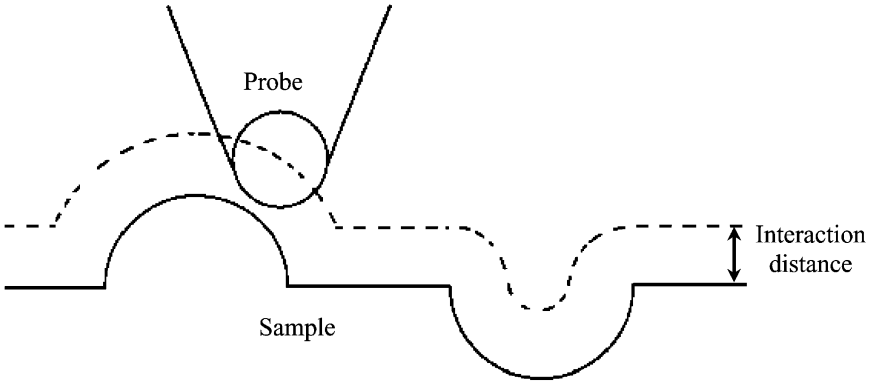


Fig. 1. Schematic representation of the basic principle of all scanning probe microscopies. The geometry of the probe and the sample-probe interaction distance conditions the resolution of the microscope and the quality of the picture (adapted from Ref. [191]).

techniques based on the measurement of other physical properties; Namely, scanning near-field optical microscopy[1] (SNOM or NSOM), where the sample is scanned by an optic fiber, measuring the photon tunnel effect or the light after near-field interactions [4,5]; scanning magnetic microscopy, where the magnetic interaction between a point of the sample and the magnetic probe is measured [6,7]; scanning ion conductance microscopy (SICM; [8]); and scanning electrochemical microscopy (SECM; [9]).

In contrast with what happened for the majority of the microscopy techniques (as well as for several other experimental techniques), only 2 years had passed since its invention when the first works in which AFM was used for the study of biological samples were published (e.g. [10]). However, the lack of reproducibility of the presented results and the refutation of the conclusions of some of these initial studies raised serious doubts on the applicability of this technique on the study of samples of biological interest. These doubts were definitively overcome in 1992, with the publication of the first reliable DNA images obtained by AFM [11]. Since then, the significant improvements obtained, both at the instrumental and sample preparation levels, led to a fast development of the atomic force microscopy of biologically relevant samples (this subject has been previously reviewed in Refs. [12–24]).

[1] This technique appeared recently coupled to fluorescence spectroscopy techniques for the measurement of energy transfer [3].

## 2. Instrumentation

Due to their global simplicity, atomic force microscopes are surprisingly small and compact instruments. Their use requires the connection to an electronic controller, a computer and (usually) two screens (in order to enable the simultaneous checking of the sample image and experimental parameters). Attending to the AFM potentialities in terms of resolution and to its reduced dimensions, it would be expectable that its isolation from any external vibration would be a major difficulty. However, the problem can be easily overcome by placing the microscope on a small solid surface, suspended by elastic cables (usually climbing ropes). Thus, the vibrations are reduced to low frequency oscillations that do not interfere with the AFM measurements.

As mentioned above, all the SPM are based on the measurement of an interaction between the sample surface and a probe located very close to it. In AFM, this interaction corresponds to the force between the atoms of the sample and those of a thin tip that scans its surface. The tip is assembled under an extremely flexible cantilever, responsible for the signal transduction (Fig. 2).

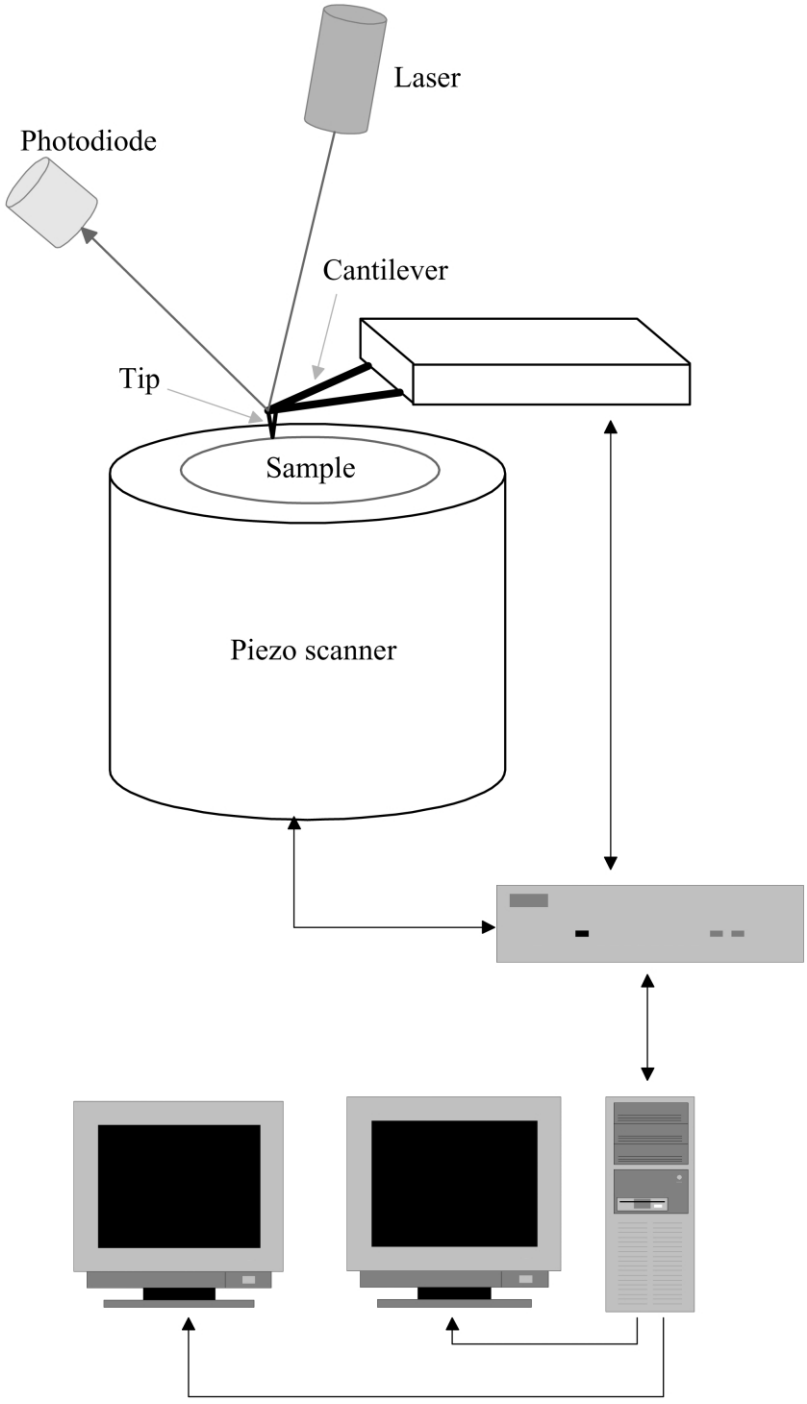


Fig. 2. Schematic representation of the components of the equipment for atomic force microscopy.

The cantilever can be simple or triangular (one or two 'legs', respectively), usually between 23- and 300-μm length, 10–30-μm width and 0.5–3-μm thick [13,25]. The most commonly used are made of silicon nitride ($Si_3N_4$), with force constants ranging from 0.01 to 100 $Nm^{-1}$. In the last few years, the use of carbon nanotubes as AFM tips has gained an increased acceptance, due to their higher resolution, small diameter, well-defined structure, mechanical robustness and unique chemical properties (for a specific review on this subject, see Refs. [26,27]; a method for the preparation of these tips are described in Ref. [28]).

Any interaction between the tip and the sample leads to a bending (or in some cases, twisting) of the cantilever. If the bending is not excessively sharp, it will be proportional to the interaction force. In order to enable the detection of any bending or twisting, the AFM uses a small laser, focused on the extreme of the cantilever (on the face opposite to the tip). The reflection of the laser beam is focused on a photodiode. It is the variation of the point of incidence of the reflected beam on the photodiode that measures any minimal bending or twisting of the cantilever and thus, the interaction of the tip with the sample. This optic mechanism enables the detection of forces usually between $10^{-7}$ and $10^{-12}$ N (e.g. [23]).

The scanning of the AFM can be obtained by a movement of the probe or of the sample itself. The sample is assembled on a piezoelectric support (piezo scanner) that besides enabling the scanning through the displacement of the sample on the *xy* plan is responsible for the movement on the *z*-axis. This mechanism has alterations on its dimensions of approximately 0.1 nm/V of applied electrical potential [29]. Thus, when the tip reaches a rise or depression on the sample, there is a change in the interaction force. Through a feedback mechanism, this change leads to an approach or removal of the sample relatively to the tip. This way, the scanning is usually carried out keeping an approximately constant distance between probe and sample, associating a *z* value to each *xy* pair. The values are registered by the computer and used for the reconstitution of a pseudo-three-dimensional picture of the sample.

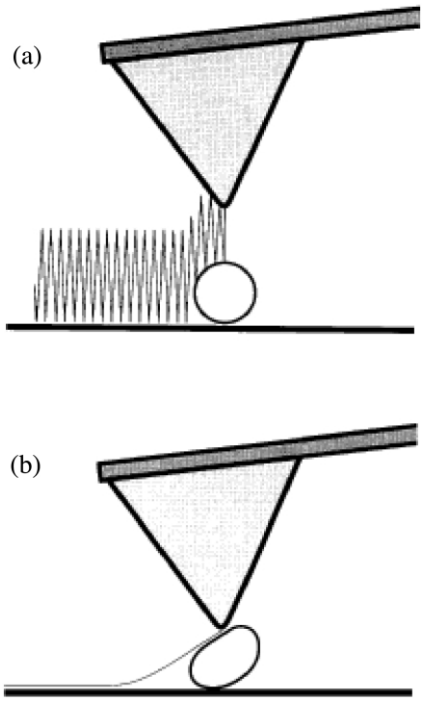


Fig. 3. Comparison between the two AFM scanning types: tapping mode (a) and contact mode (b). Usually, the first one can minimize sample deformation (adapted from Ref. [60]).

Beside the traditional contact mode (described above), the latest generations of atomic force microscopes also use another scanning mode called tapping mode [30] or MacMode (depending on the microscope brand). This mode uses an acoustic drive of the tip with pre-defined height baseline, frequency and amplitude (Fig. 3). The alterations in these parameters, due to an interaction with the sample are a function of its topography. In the same way as with the contact mode, this data is used by the computer to obtain a pseudo-three-dimensional image of the sample surface. Despite the difficulties in defining experimental parameters, such as amplitude (up to 20 nm) and frequency (normally between 10 and 40 kHz, for measures in aqueous solution, but ranging upto 650 kHz [31]), in most studies the tapping mode leads to higher resolution images, minimizing the damages in soft samples. The phase shifts of the tip oscillation relative to the driving signal,

induced by the interaction with a heterogeneous sample, can also be used to obtain an alternative mapping of its surface (named, phase imaging [32]). Recently, a more complex analysis of the tapping oscillation (high-Q dynamic force microscopy) has also been used on the imaging of biological samples [33]. The complex physical and mathematical models underlying the different sampling modes and imaging processes have been recently (and extensively) reviewed by García and Pérez [34] and are out of the scope of the present work.

The possibility of carrying out measurements in aqueous solution is extremely important for the study of biological samples with AFM. In atomic force microscopy studies, this objective is achieved using a fluid cell. These devices differ between manufacturers but, in general, they use an o-ring around the sample, placed on the impermeable head of the piezo scanner (the remaining part of this mechanism can be damaged by humidity). The upper part of the fluid cell is formed by a surface transparent to the laser beam that together with the o-ring seals the sample and its environment. This device has input and output lines for fluid substitution, and can be purchased with an extra entrance for direct temperature control.

The development of atomic force microscopes with a high speed at which good quality successive images can be recorded has a key importance. A new AFM apparatus, recently described [31], generates movies with 80 ms frames, enabling the following of faster biological events.

As it happened with many other experimental techniques, there was an attempt to combine the atomic force microscope with other types of microscopy. Some important results were obtained by AFM coupled with fluorescence techniques [35–38], optic microscopy [39], scanning ion conductance microscopy [40] or scanning electrochemical microscopy [41].

## 3. Sample preparation

Until now, one of the main limitations for the application of atomic force microscopy to the study of biological relevant samples has been the sample preparation. While the study of more rigid materials can be carried out with high resolution and no damage to the sample on the study of the majority of the biomaterials, the sample can be deformed or dragged away by the tip. This effect is even more evident on studies in aqueous solution, where sometimes the movement of the tip is enough to induce the solubilization of the molecule under evaluation [42]. There is no universal solution to deal with these problems, making it necessary to develop a specific method for each type of system.

On the study of biological samples, it is important to find a method that binds the sample to the substrate, so that the binding is strong enough to avoid the dragging by the tip but simultaneously, does not cause structural alterations in the system. These alterations can also result from other factors, such as the collapse that can be induced in a sample by the change of surface tension during drying or freezing, or the topography distortion caused by any type of coating. Regardless, the choice of the method for sample preparation, it is equally necessary to minimize the force applied in the measurement and to choose the scanning mode that causes minor sample alterations (in most of the cases tapping mode has shown to be the most appropriate).

As in other types of microscopy, usually the biological sample has to be deposited on a solid substrate (that will be attached to the base of the microscope). The ideal substrate has to be smooth at the atomic level (in order to avoid incorrect interpretations of the topography of the sample) and it should have a high affinity for the studied molecules (allowing its adsorption, but keeping them atleast partially functional). In the majority of the situations, the adsorption is obtained through physical interactions (electrostatic, polar or van der Waals interactions). In the process of adsorption of a biomolecule, almost all these interactions can be adjusted by changes in pH, ionic strength or solution composition. Thus, the variation of these parameters is one of the first processes for optimizing adsorption conditions [43].

The most common substrates for AFM studies are glass and mica. Muscovite (the type of mica normally used) is constituted by layers of $(Si,Al)_2O_5$ ionically bound to a central layer of

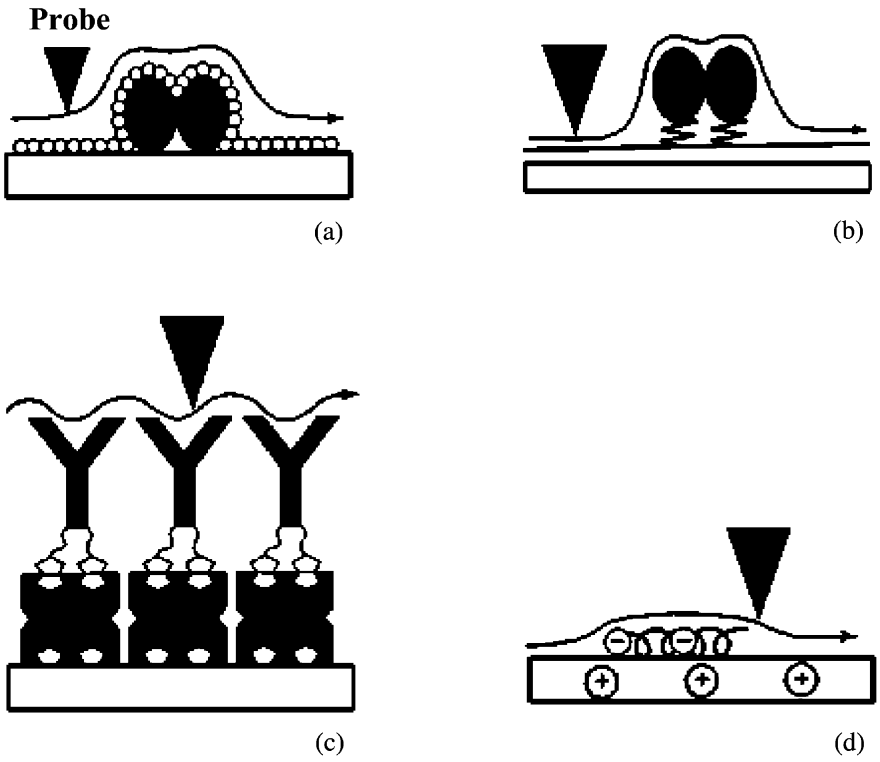


Fig. 4. Methods used for immobilization of biological samples of difficult preparation for AFM studies. (a) Coating of biomolecules with a thin metal layer. (b) Covalent binding of a biomolecule to a chemically activated substrate. (c). Use of molecules with great affinity between themselves, through the covering of the substrate with streptavidin, to which the molecule in study, derivatized with biotin, is bound. (d). Electrostatic immobilization through the use of a substrate with a net charge opposite to the biomolecule (adapted from Ref. [17]).

$Al_2(OH)_2$. The negative net charge is compensated by potassium ions. While glass surfaces present irregularities on the nanometer-range and needs a great care in its cleaning, in order to keep it hydrophilic, mica can be easily cleaved (by the $K^+$ layer) with a simple scotch tape, producing a clean, hydrophilic and smooth surface at the atomic level. In aqueous solution, both substrates present negative surface charges, allowing an easy adsorption of positive surface charge molecules. The adsorption of negative surface charge molecules is also easily obtained through the presence in solution of divalent cations that will act as intermediates in the interaction between the two surfaces. Extremely smooth gold surfaces (prepared over mica, which is removed only at the moment of use) are also chosen as substrate [44].

If the substrate is not readily used after its preparations, when exposed to air it will be quickly covered with hydrocarbons, which will modify the sample adsorption and the AFM measurements. The same happens when the AFM tip adsorbs these hydrocarbons. The organic contamination of tip and substrate can be eliminated by exposure to UV radiation. All the aqueous solutions to be used in the measurements or cleaning of the equipment must be prepared with ultra-pure water (prepared in a Millipore system or similar), which contains lower levels of hydrocarbons and other contaminants than conventional bi-distilled water.

When the preparation of samples by the processes described above is impossible, difficult or shows low reproducibility, more complex processes become necessary (Fig. 4). One of the most

commons is to act over the substrate and/or molecule to deposit, in order to bind them chemically. This can be achieved by direct covalent binding or through the functionalization of the molecule under study and/or substrate with groups that bind with great efficiency (e.g. biotin–streptavidin, substrate-bound antibodies specific for the molecule to be studied). The coating of samples of difficult stabilization with a metal film is widely used in other microscopy techniques (e.g. scanning electron microscopy), and can also be used for AFM sample preparation. Besides stabilizing the sample, the coating becomes resistant to sample deformation, enabling the use of scanning conditions normally reserved to the study of rigid samples. However, when this process is used, one of the biggest potentialities of atomic force microscopy is lost: the direct visualization of the sample without the need of coating, fixing or contrast inherent to electron microscopies. Even the thinnest metal film of a coating induces alteration on the dimensions and topography of the sample, leading to possible experimental artifacts that can be confused with the expected molecular structures [17].

Cryo-AFM, is an instrumental innovation described for the first time in 1991, in which the samples are studied at low temperature (the best results were obtained using liquid nitrogen vapor), which overcomes many of the difficulties and limitations of the study of biological samples with the atomic force microscope [45–47]. With the increase of sample rigidity at low temperatures and the reduction of thermal fluctuations of the cantilever, the resolution obtained is considerably higher than that achieved with the AFM at room temperature. Obviously, this methodology also implies that the studies are not carried out in physiological conditions, losing one of the main advantages of AFM over other types of microscopy.

## 4. Applications

### *4.1. Nucleic acids*

Immediately after the invention of the atomic force microscope, DNA was the subject of some of the first attempts of AFM application to the study of biological samples. These studies had soon generated a great interest in the scientific community, motivated in special by the hope of reaching a resolution good enough to distinguish specific nucleotides. Today, the goal of enabling this incredibly fast method for sequencing is still far from being achieved. The credibility of these initial works was, in part, shaken by the discovery that some of the structures described as DNA in STM studies, were in fact experimental artifacts [48].

The work of Bustamante et al. [11] marked a significant turn on the studies of nucleic acids. In this work, deposited carbon tips were used to study DNA in air, adsorbed to the substrate (mica) due to the presence of $Mg^{2+}$. However, the topographical information showed a width wider than expected, as well as a substantially lower height. The first artifact was possibly due to convolution with the radius of the tip, increasing the apparent width. The error on the DNA height measurements (also reported in several other studies) could have resulted, in part, from the compression of the DNA by the tip and covering of the remaining surface of the substrate by magnesium acetate.

The use of improved equipment and new methodologies [49–58], and the study of nucleic acids by AFM in aqueous solution [59] allowed the proliferation of more elaborated studies, including the observation of DNA degradation by the enzyme DNase I [60], conformational analysis of the double-helix [61], observation of the transcription by a RNA polymerase [62], visualization of the DNA bending induced by an integration host factor [63], direct measurement of DNA mechanical properties and their modulation upon the binding of small molecules [64], structural analysis of chromosome damage induced by radiation [65,66], imaging of unwinding activity of duplex RNA by a helicase [67], mapping of mismatched base pairs in DNA molecules [68], location of protein binding sites on DNA [69,70] and RNA [71] chains, and the observation of the translocation and cleavage of DNA by an endonuclease [72].

### 4.2. Supported membranes

The study of phospholipid membranes is one of the field, where the application of atomic force microscopy to the study of samples of biological interest presents a higher potential (for recent reviews, see [73–75]). Through the preparation of supported membranes, large areas of uniform membrane are achieved in a reproducible way. The deposition of bilayers or multilayers using a Langmuir–Blodgett trough (equipped for this effect), is the method that leads to better quality samples. For experimental convenience, many authors have tried the direct fusing of vesicles (usually small unilamellar vesicles, SUV) with the substrate or an intermediate method, where the first monolayer is deposited in a Langmuir–Blodgett trough, and the following ones by vesicle fusion (e.g. [18]). Regardless of the method of preparation, the handling of the samples has to be extremely careful, in order to prevent membrane disruption or any other undesirable alteration.

The AFM studies involving planar membranes can be divided in to two main groups: those which focus on the membrane itself (e.g. [75–81]) and those which deal with the insertion of other molecules in the membrane (e.g. [82–86]). Lipid membranes can present a complex behavior, with phase changes and phase coexistence (for review, see e.g. [87]). They present distinct phospholipid organizations, leading to the existence of several physical parameters that can be resolved by AFM (e.g. bilayer thickness and its spacing, viscosity, formation of bidimensional arrays of phospholipids, dimensions of membrane domains and their shape). On the study of the surface organization of some molecules, the atomic force microscope can reach the resolution of individual phospholipid polar groups [88,89] and fatty acid salts [90]. However, this resolution has not been obtained yet for the majority of the lipid systems. Other studies developed in this field include the imaging of the membrane lesions resultant from the incorporation of an antibiotic [91], degradation of lipid bilayers by phospholipase $A_2$ [92], preparation of planar membranes from high density lipoproteins [93], domain interdigitation caused by ethanol [94] and phase transition in fatty acid monolayers [95]. More recently, the mechanical properties of membranes have also been studied (e.g. [96–99]).

### 4.3. Proteins

The natural immobilization of membrane proteins, allied to the absence of other especially appropriate techniques for the study of these biomolecules, has made them the subject of the majority of the protein studies by AFM. The isolated protein that have been studied with relative success include muscular fibers of actin [100] and myosin [101], and β-amyloid fibrilization [102,103]. For soluble proteins, if an efficient stabilization process is not attained they become almost unsuitable to resist to the scanning by the probe. However, some important results have been achieved with soluble proteins deposited in layers (e.g. [104]), by direct adsorption (e.g. [105–107]), using cryo-AFM (e.g. [108]) or in the majority of the cases by chemical bonds between molecule and substrate (e.g. [107,109]). Protein–protein association constants have been quantified [110,111] and dynamic events can be monitored [112] by AFM. Crystal growth studies are not usually hampered by these limitations [113–115].

The study of membrane proteins, that creates so many problems for most of the techniques used for structural characterization, acts as an increased advantage in AFM works. Besides providing an uniform surface, the incorporation in the membrane stabilizes the protein structure and limits its number of possible orientations. The advantages of these systems have even allowed lateral resolution of approximately 0.5 nm and vertical resolution of approximately 0.1 nm [23,116].

The best membrane protein images have been produced through crystallization in two-dimensions (for review, see [117]). These membrane protein monolayers, correctly oriented and showing a large surface concentration, intercalated with phospholipids and detergent, are prepared from a large initial amount of protein or natural membranes, from which contaminants and excess of lipids are extracted (using detergents). This process origins micelles, which are converted into destabilized liposomes. The crystallization of the sample in the desired substrate is finally obtained

through the reduction of the detergent concentration. Obviously, the ideal conditions for this process have to be carefully optimized, varying for each case. Some of the membrane proteins already studied by AFM in crystal form, include Aquaporine-1 [118], porine OmpF [119], calcium-ATPase [120] and cholera toxin [121,122].

Beside the works involving proteins in two-dimensions crystals, many other studies of AFM have been done with membrane proteins deposited in planar membranes, without their crystallization. In some of these works, resolutions equivalent to those achieved with crystallized proteins were obtained, as it can be seen in the study of the $F_1F_0$ complex of ATP synthase [123]. The use of membrane proteins in this form also allows studies that would be impossible to carry out with crystallized proteins, such as the studies of the aggregation of gramicidin in membranes [124] or photosynthetic protein distribution in deposited membranes with the same composition as native membranes [125].

The studies of membrane proteins directly on the surface of a cell, without the necessity of their isolation will be discussed later.

### *4.4. Polysaccharides*

As it happens with many other techniques, the number of studies of polysaccharides by AFM is quite lower than those dedicated to other biomolecules, despite the important functions they play in nature [126]. Some of these AFM studies have been dedicated to the elucidation of the structure of bacteria [127], plant [128–132] and fungi polysaccharides [133], as well as of densely glycosilated peptides [134] and proteoglycans [82]. In this area, one of the most interesting recent studies involves the identification of individual polysaccharide molecules in solution [135], a feature that cannot be carried out by the other available techniques. There are also some interesting non-topographical studies involving polysaccharides, as it will be discussed later.

### *4.5. Cells and virus*

The possibility of the AFM to be operated under physiological conditions, without submitting the sample to fixation or any other chemical preparation, allowed its use on the observation of living cells. Under appropriate experimental conditions, these cells remained viable during extensive periods of time, without damages caused by the scanning. This imaging allows the detection of structures present in the cellular surface, with dimensions down to 20 nm. These studies lead to images of lower quality, when compared with those obtained on other AFM studies or on studies using the same systems but other microscopy techniques. However, they have the advantage of being made in conditions much closer to the natural ones.

Despite the fact that the atomic force microscope has been mainly used on cell surface observation, it has also been applied to the study of intracellular structures, without the need of any contrast method. These studies have focused specially on the visualization of rigid intracellular structures, such as the actin filaments [136], the whole cytoskeleton [137,138], the interior structure of synaptic vesicles [139], and sub-surface features on cardiac myocytes [140] and sperm [141].

Among the cellular surface studies by AFM (for a review on microbial surfaces, see [142]), some of them should be emphasized, namely, the observation of the redistribution of blood platelets constituents during their activation [143], exocytose of viral particles in an infected cell line [144], conformational alterations induced in the nuclear membrane pores [145], membrane proteins expressed in oocytes [146], lectin-labeled lymphocytes [147], polymer adsorption onto human buccal cells [148], neurite mast-cell communication [149], enzymatic degradation of membrane proteins in epithelial cells [150] and, structure of hepatic gap junctions [151]. Some of the most interesting AFM cell studies were carried out with erythrocytes; Namely, the imaging of mixed layers of group A and 0 erythrocytes with a contrast based only on the measured strength of a receptor–ligand pair (using a tip functionalized with a specific lectin [152]), the identification of the protrusions induced by *Plasmodium falciparum*-infection [153], the imaging of shear stress alterations [154], the demonstration of the formation of membrane domains and pores induced by the presence of lanthanide cations [155], the imaging

of regular lattices on the membrane [156,157], and the characterization of the exovesicles released from the erythrocyte membrane [158].

Some AFM studies have also focused on the observation of virus, such as pox virus [144], bacteriophagus T4 [159] and tobacco mosaic virus [160,161].

As the AFM 'touches' the cell surface, not reaching a true direct visualization, the risk of ambiguity on the identification of some structures exists. This is especially important for the observation of the complexity of cellular surfaces, creating the need for additional means of identification. One of the ways to overcome this difficulty has been through the coupling of the AFM with another instrument, usually an optic microscope [39].

### *4.6. Non-topographical applications*

In general, the atomic force microscopy studies can be divided in topographical and non-topographical applications. In the first group, we can include all the studies already discussed, where the main objective consists in getting an image of the sample surface, for its structural or dynamic characterization. The non-topographical applications are one of the most promising and interesting areas of this microscopy (for reviews on detailed topics, see [162–168]), allowing the study of inter and intramolecular forces, as well as the manipulation of the sample following several approaches that were virtually impossible until the development of these methodologies.

Through the optimization of the scanning conditions, the tip of the microscope can be used, not only as a probe, but also as a tool for sample manipulation, allowing its cut, dragging, dissection or conformational alteration. These methods have already been successfully applied to the study of cells [151,169,170], virus [160], proteins [171,172], lipoproteins [93] and planar lipid membranes [96].

Many of the non-topographical applications of the AFM appeal to a more quantitative use, through the measurement of surface forces, elasticity, adhesion, rigidity, friction or viscosity (e.g. [173–179]). Some of these properties are measured using force vs. distance plots or force vs. time plots (e.g. [174,178,179]). In these methodologies, the AFM (or instruments built through its modification) does not carry out any kind of scanning, measuring only the interactions between the tip and the surface of the sample.

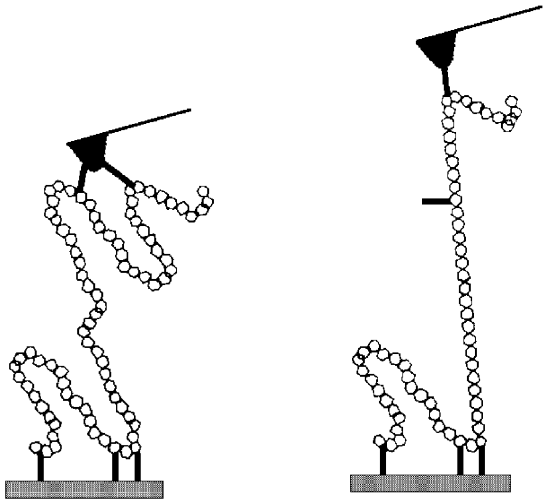

Fig. 5. Direct measurement of the force necessary to unfold one biomolecule through its covalent binding to the tip of the AFM and to the substrate (adapted from Ref. [182]).

The improvement of the studies using force vs. distance plots lead to the creation of a new experimental possibility, named single-molecule force spectroscopy (SMFS). This method allows what until now was considered impossible: the measurement of mechanical properties of single molecules, with a sensitivity in the range of 1 pN and a spatial resolution below 1 nm [180]. For this utilization, derivatized tips are used in order to bind easily to the molecule under evaluation. The sample is analyzed following a scanning pattern similar to tapping mode, until the formation of the bond. Then, it becomes possible to measure and interpret [181] the force necessary to break the bond or to modify the conformation of a molecule simultaneously bound to the substrate and to the tip (Fig. 5). The SMFS technique has already enabled the direct determination of the force necessary to break an isolated covalent bond [182], an antigen–antibody interaction [183] and

the bonds between DNA base pairs [184], as well as the force necessary for protein unfolding [84,171,185] or conformational alteration of polysaccharides [92]. A detailed quantitative comparison of mechanical and chemical unfolding of a single protein was possible [186,187].

The outstanding sub-nanometer resolution of the AFM in the *z*-axis also enables another form of non-topographical application. Placing the tip of the microscope over a protein, it is possible to register the time variation of its 'height', without any scanning. The changes on the standard fluctuation of this parameter, in the presence and absence of a key reagents can be used to directly follow the activity of a single enzyme [188], and primary light events in bacteriorhodopsin [189]. A similar procedure was already successfully applied to the measurement of the activity of an ionic channel [190].

Considering the importance for the understanding of several biological systems of observing the response of a molecule to an externally applied perturbation, Wallace et al. [38] have recently discussed the use of an AFM-related apparatus for the manipulation of a single molecule with a precise mechanical load, with simultaneously monitoring of the repercussions on its fluorescence properties.

## 5. Conclusions

The potentialities of the atomic force microscopy make it a tool of undeniable value for the study of biologically relevant samples. In this field, it should be emphasized its potential to: (i) image the structure of biomolecules or bio-surfaces with molecular (or even submolecular) resolution; (ii) study samples in solution, air, vacuum or even liquid nitrogen, within a vast range of temperatures; (iii) carry out studies in physiological conditions, with the possibility to change parameters such as pH or ionic strength, which allows to follow in situ the real time living dynamics of events in biomolecules or cells; (iv) measure local chemical, physical and mechanical properties of surfaces (charge density, adhesion, rigidity, viscosity, elasticity) and intermolecular forces within a spatial resolution below 1 nm; and (v) manipulate single molecules. This expansion of the use of atomic force microscopy can be registered by the fast growth of publications, where the AFM is used for the study of biologically relevant samples [16], in such areas as Biophysics, Biochemistry, Microbiology, Structural Biology, Biomaterials and Medical Sciences.

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