Probing molecular recognition sites on biosurfaces using AFM

Vincent Dupres¹, Claire Verbelen, Yves F. Dufrène*

Unité de Chimie des Interfaces, Université Catholique de Louvain, Croix du Sud 2/18, B 1348 Louvain-la-Neuve, Belgium

Received 18 August 2006; accepted 7 November 2006

Abstract

Knowledge of the molecular forces that drive receptor–ligand interactions is a key to gain a detailed understanding of cell adhesion events and to develop novel applications in biomaterials science. Until recently, there was no tool available for analyzing and mapping these forces on complex biosurfaces like cell surfaces. During the past decade, however, single-molecule atomic force microscopy (AFM) has opened exciting new opportunities for detecting and localizing molecular recognition forces on artificial biosurfaces and on living cells. In this review, we describe the general principles of the AFM technique, present procedures commonly used to prepare samples and tips, and discuss a number of applications that are relevant to the field of biomaterials.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Atomic force microscopy; Biosurfaces; Molecular recognition; Single molecules; Functionalized tips; Receptor–ligand interactions

Contents

1. Introduction ...................................................... 1
2. Principles of AFM .............................................. 2
3. Samples .......................................................... 3
4. Tips ............................................................... 3
5. Molecular recognition forces .................................... 4
6. Molecular recognition mapping .................................. 5
7. Conclusions .................................................... 6
Acknowledgments ................................................. 7
References ........................................................ 8

1. Introduction

Molecular recognition between specific receptors and ligands plays a central role in life science. Examples of such specific interactions are those between complementary strands of DNA, enzyme–substrate, antigen–antibody, lectin–carbohydrate, and cell-adhesion proteins. In biomaterials science, probing receptor–ligand interactions is essential to gain a detailed understanding of cellular events such as cell adhesion [1,2] and to control cell responses [3,4]. During the past decades, tremendous progress has been made in characterizing biomolecular forces, using techniques like the osmotic stress method [5], the surface forces apparatus [6], magnetic beads [7], optical tweezers [8] and the biomembrane force probe [9]. Yet, these techniques do not offer lateral resolution and are generally not suited for mapping recognition sites on living cells.

The advent of atomic force microscopy (AFM) has opened a wide range of novel possibilities for exploring
biosurfaces, in terms of structure, properties and biomolecular interactions. While AFM imaging is used for generating three-dimensional topographic views of biological specimens at high resolution and under physiological conditions [10,11], single-molecule force spectroscopy allows researchers to measure biomolecular forces with piconewton sensitivity [12,13]. These nanoscale measurements provide new insight into the structure–function relationships of biosurfaces and contribute to the development of novel biotechnological and biomedical applications. In this paper, we discuss the methodology of AFM force spectroscopy and highlight applications offered by the technique in biomaterials research, with an emphasis on molecular recognition studies.

2. Principles of AFM

The basic idea behind AFM is that three-dimensional images of surfaces can be obtained by sensing the force between a sample and a sharp tip mounted at the end of a soft cantilever, without using an incident beam as in classical microscopies. To this end, the specimen is mounted on a piezoelectric scanner, which allows three-dimensional positioning with sub-nanometer accuracy while the force is monitored with piconewton sensitivity by measuring the deflection of the cantilever. The deflection, or vertical bending of the cantilever, is usually detected using a laser beam focused on the free end of the cantilever and reflected into a photodiode. AFM cantilevers and tips are typically made of silicon or silicon nitride using microfabrication techniques.

A number of different AFM imaging modes are available, which differ mainly in the way the tip is moving over the sample. In the so-called contact mode, the AFM tip is raster scanned over the sample while the cantilever deflection, thus the force applied to the tip, is kept constant using feedback control. In tapping mode, also called dynamic or intermittent mode, an oscillating tip is scanned over the surface and the amplitude and phase of the cantilever deflection of the same amount as the sample movement. In fact, different behaviors may occur in the cantilever deflection of the same amount as the sample movement. In fact, different behaviors may occur in the contact region, depending on the sample stiffness. On a hard and non-deformable surface, a vertical line is recorded (label A); while on a soft surface, sample indentation will occur, leading to a different shape (label C). Analyzing this behavior with appropriate theoretical models may provide direct information on the sample elasticity. Upon retracting the tip from the surface, the curve often shows a hysteresis referred to as the adhesion “pull-off” force (label D), which can be used to

![Fig. 1. Principle of AFM force spectroscopy: the different portions of a force vs. distance curve (see text for details).](image-url)
estimate the surface energy of solids or the binding forces between complementary biomolecules. In the presence of long, flexible molecules, an attractive force, referred to as an elongation force, may develop non-linearly (label D'). Further details on the principles of force spectroscopy can be found in the excellent review by Butt and co-workers [20].

3. Samples

Firm immobilization of the sample onto a supporting surface is a crucial prerequisite for successful AFM imaging and force spectroscopy analyses. Mica, glass and silicon oxide have proved to be excellent supports for biomolecules. Particularly, muscovite mica has been the most widely used material for immobilizing biological species, including double-stranded DNA, protein arrays or supported lipid films. This non-conducting layered mineral can be easily cleaved using adhesive tape to produce clean, atomically flat surfaces. Membrane proteins that form stable two-dimensional crystals are easily immobilized by simple adsorption [21,22] and very well-suited for high-resolution imaging [23]. Positively charged proteins, such as lysozyme, can be simply adsorbed to mica, glass or silicon oxide surfaces since these are negatively charged at neutral pH [24]. DNA molecules can be imaged when bind onto mica in the presence of certain divalent cations (Ni$^{2+}$, Co$^{2+}$, Zn$^{2+}$ or Mn$^{2+}$) [25,26]. In certain cases covalent attachment of biomolecules may sometimes be required to allow imaging [27–29]. More details on sample preparations can be found in review articles [21,22,30–32].

How about living cells? A simple preparation method for animal cells is to let them spread and adhere to solid surfaces [33,34]. Coating the support with adhesion proteins such as collagen may be used to enhance immobilization [35]. Chemical fixation using cross-linking agents such as glutaraldehyde may be required either to prevent cell damage or detachment by the scanning tip or to obtain high-resolution images [36]. Unlike animal cells, microbial cells like bacteria and yeasts do not spread on solid surfaces, meaning immobilization by simple adsorption is not appropriate. Strong attachment may be achieved either by pretreating the support with polycations [37] or by bonding the cells covalently to the support [38]. Alternatively, cells can be mechanically trapped in an agar gel or in a porous membrane. In the first approach, the agar gel is used as a soft, deformable immobilization matrix, thereby allowing direct visualization of growth processes [39]. In the second method, spherical cells are trapped in a polymer membrane with a pore size comparable to the dimensions of the cell, allowing repeated imaging without cell detachment or cell damage [40,41].

4. Tips

Probing molecular interactions and surface properties by force spectroscopy often requires the use of functionalized tips. Chemically functionalized tips can be obtained by modifying their surface with organic monolayers terminated by specific functional groups (e.g., OH, COOH or CH$_3$). The most common method is based on the formation of self-assembled monolayers (SAMs) of alkane thiols on gold surfaces. The procedure involves coating microfabricated cantilevers with a thin adhesive layer of Cr or Ti, followed by another thin layer of Au, and immersing the coated cantilevers in dilute ethanol solutions of the selected alkane thiol.

Modifying tips with chemical groups enables quantitative measurements of cell surface properties. For instance, Ahimou and co-workers [42] used tips bearing ionizable carboxyl groups to probe the surface charges of living yeast cells at the nanometer level. Force–distance curves were strongly dependent on pH due to cell/tip electrostatic properties, and allowed to assess the local cell surface isoelectric point. Using a similar approach, hydrophilic and hydrophobic tips were used to map the surface hydrophobicity of fungal spores [43].

Molecular recognition studies imply functionalizing the tips (and supports) with relevant biomolecules. Here, several important issues must be considered: (i) the forces which immobilize the molecules should be stronger than the intermolecular force being studied; (ii) the attached biomolecules should have enough mobility so that they can freely interact with complementary molecules; (iii) the contribution of non-specific adhesion to the measured forces should be minimized; (iv) attaching biomolecules at low surface density is recommended in order to ensure single molecule detection; (v) site-directed coupling may be desired to orientate all the interacting molecules in the same way.

Immobilization strategies commonly used for making such biological tips are illustrated in Fig. 2. Note that these approaches are also valid for preparing flat supports. Using the non-specific adsorption of biotinylated bovine serum albumin (BBSA), it was possible to measure the interaction between biotin and avidin (or streptavidin) down to the single molecule level [44–46]. The adsorbed BBSA layer can actually be further reacted with avidin/streptavidin to attach biotinylated molecules [47,48].

A second approach relies on the strong binding of thiols on gold-coated tips. While proteins, oligonucleotides and carbohydrates that bear thiol groups can directly be attached on the gold surfaces [49,50], biomolecules can also be covalently attached onto SAMs of functionalized alkanethiols on gold, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) [51]. In this context, an interesting approach is to mix long chain alkanethiols with COOH terminal functions in a matrix of shorter OH-terminated alkanethiols, which ensures a certain mobility of the attached biomolecules and minimizes non-specific adsorption [52,53]. Importantly, it is possible to orientate all biomolecules in the same way by attaching recombinant histidine-tagged proteins onto an AFM tip coated with nitrilotriacetate (NTA)-terminated...
alkanethiols [48,54]. This coupling approach offers the advantage of allowing an optimal exposure of the C-terminal or N-terminal domains, but is limited by the rather low binding strength of the NTA–His bond.

A third approach is to covalently anchor biomolecules on silicon tips using various amine-functionalization procedures [55,56]. The amino-terminated surfaces are reacted with a crosslinker which provides the ligands with motional freedom and prevents their denaturation. Crosslinkers typically carry two different functional ends, e.g. an amine reactive NHS group on one end, and 2-pyridyl-dithiopropionyl (PDP) or vinyl sulfone groups, which can be covalently bound to thiols, on the other.

For cell adhesion studies, animal and microbial cells can be attached onto AFM cantilevers, allowing microscopists to probe various types of cellular interactions. Microbial cells have been immobilized by using glutaraldehyde treatments to create covalent cross-linking between cells and tips [57] or by attaching single cells with a small amount of glue [58]. However, these treatments are likely to affect the structure and properties of the cell surfaces. Alternatively, bacteria can be physically adsorbed onto poly-L-lysine-coated glass beads followed by attaching the cell-coated beads to a cantilever by using a small amount of epoxy resin [59]. Another elegant approach is to attach individual cells to an AFM cantilever via lectins such as wheatgerm agglutinin or concanavalin A (Con A) which then allows to measure the specific adhesion forces between two adjacent cells [60] or between cells and immobilized ligands [61,62].

5. Molecular recognition forces

Following pioneering work on the biotin/avidin(streptavidin) interaction [44,45], a variety of ligand–receptor forces have been measured at the single molecule level including those between antibody–antigen [55,63], complementary strands of DNA [49,64,65], carbohydrate–carbohydrate [51] and lectin–carbohydrate [66]. Of particular interest in biomaterials science is the measurement of the specific forces associated with cell adhesion proteins, such as cadherins [67], integrins [61,62], selectins [68] and bacterial adhesins [48]. Clearly, knowledge of these adhesion forces contributes to refine our understanding of the molecular basis of cell–cell and cell–material interactions.

As we have seen before (Section 2), molecular recognition studies imply recording force curves between modified tips and supports and then assessing the unbinding force between complementary receptor and ligand molecules from the adhesion ‘pull-off’ force observed upon retraction (Fig. 1). The measured unbinding forces are typically in the 50–400 pN range, depending on the experimental conditions, i.e. number of interacting molecules and the loading rate (see below for more details on this parameter). As a rule, control experiments should always be carried out to demonstrate the specificity of the measured unbinding forces, which is best achieved by using block experiments in which the receptor sites are masked by adding free ligands or by exploiting genetic mutation. A large number of force curves (~1000) should be recorded using many independent tips and samples to establish the validity and reproducibility of the forces, the final data being usually expressed as adhesion force histograms. With fragile samples like most living cells, avoiding tip contamination or damage may be a crucial issue. This problem can be limited by using non-adsorbing surface chemistries for modifying the tips (see Section 4), by recording force curves immediately after the tip is engaged on the target surface and see how they change with time, and by triggering the maximum contact force.

To guarantee single molecule interactions, it is often useful to dilute the surface density of attached biomolecules (Section 4) and to modulate the contact force and/or the contact time between tip and sample.

A widely investigated family of receptor–ligand interactions are those between lectins and complementary

![Fig. 2. Strategies commonly used for modifying AFM tips for single molecular recognition studies: physisorption of proteins such as biotinylated BSA, chemisorption of alkanethiols on gold and covalent coupling of silanes on silicon oxide. Adapted with permission from [85].](image)
carbohydrates, which mediate a variety of cellular interactions and play roles in the control of various processes in living organisms such as initiation of infection, promotion of symbiosis, control of differentiation, and organ formation [69,70]. For instance, the plant lectin Con A has remarkable aggregating properties resulting from the specific binding with α-mannosyl and α-glucosyl groups. To probe this interaction at the single molecule level, we recorded force curves between tips modified with oligoglycose hexasaccharides and supports modified with Con A, using a loading rate of 4000 pN/s. As shown in Fig. 3, most retraction curves showed single unbinding forces of \( \sim 100 \text{ pN} \), together with non-linear elongation forces reflecting the stretching of the long, flexible spacer [50]. Such adhesion forces were not observed when the measurements were performed in the presence of the blocking agent mannose or with hydroxyl-terminated tips, indicating that they originated from the specific interaction between individual lectin and carbohydrate molecules.

Carbohydrate tips were also used to gain insight into the aggregation of the yeast *Saccharomyces carlsbergensis* which is mediated by specific interactions between cell surface lectins and mannose residues [71]. The force curves recorded between yeast cells in aggregating conditions and the modified tips using a loading rate of 4000 pN/s showed single or multiple unbinding forces with a mean magnitude of \( \sim 100 \text{ pN} \), which is similar to the value obtained for Con A. In the presence of mannose, no/little adhesion was detected, indicating that mannose had blocked the lectin receptor sites. Further controls were carried out using non-aggregating cells; here again, the curves showed poor adhesion, which lead us to conclude that the measured 100 pN force reflected the specific interactions involved in yeast aggregation.

Another area where molecular recognition is crucial is bacterial pathogenesis, where the infectious process is generally initiated by the interaction between cell adhesion molecules on the bacterial surface, referred to as adhesins, and specific ligands on the host cell surface. An example of this is found in tuberculosis which is caused by the infectious agent *Mycobacterium tuberculosis*. *M. tuberculosis* is known to adhere to heparan sulfates on epithelial cells via the heparin-binding haemagglutinin adhesin (HBHA). To explore the binding forces of individual HBHA, force curves were recorded between HBHA-modified tips and heparin-coated supports [48]. The adhesion force histogram obtained using a loading rate of 10,000 pN/s revealed a bimodal distribution with average rupture forces of 50 and 117 pN, attributed to one and two binding events between HBHA and heparin. The specificity of the measured interaction was confirmed by showing a dramatic reduction in the number of adhesion events when working in the presence of free heparin or with a bovine serum albumin-coated tip instead of a HBHA-tip. This work shows that single-molecule force spectroscopy is a powerful tool to gain insight into the molecular mechanisms of cell adhesion processes.

Unbinding forces between receptors and ligands depend on the loading rate, i.e. the rate at which the force is applied to the bond [9,47,72,73]. Theory predicts that if only one single sharp energy barrier governs the dissociation process, unbinding forces should increase linearly with the logarithm of the loading rate. Dynamic force spectroscopy analyses have confirmed this view for a variety of

---

**Fig. 3.** Unbinding forces of single lectin–carbohydrate interactions. Force–distance curves recorded between hexasaccharide-terminated tips and concanavalin A-functionalized supports revealed adhesion forces of \( \sim 100 \text{ pN} \) attributed to the rupture of single lectin–carbohydrate complexes [50]. A drawing of the experimental set-up is also provided.

Please cite this article as: Dupres V, et al. Probing molecular recognition sites on biosurfaces using AFM. Biomaterials (2006), doi:10.1016/j.biomaterials.2006.11.011
receptors, including selectins [47], antibodies [63] and nuclear pore proteins [73]. Multiple linear regimes indicate that several barriers are present in the energy landscape [9]. Interestingly, kinetic parameters on the unbinding process can be extracted from such dynamic measurements, including length scales and relative heights of energy barriers and dissociation rate constants. By way of example, Fig. 4 shows data obtained for the antigen unbinding forces of individual Fv fragments of anti-lysozyme antibodies [53]. The plot of unbinding force vs. log (loading rate) clearly revealed two distinct linear regimes with ascending slopes, suggesting multiple barriers were present. Further, the kinetic off-rate constant of dissociation obtained by extrapolating the data of the low strength regime to zero force could be estimated and found to be in the range of values obtained by surface plasmon resonance.

6. Molecular recognition mapping

A unique feature of AFM is its ability to map the distribution of individual binding sites with nanoscale resolution, using either adhesion force mapping or dynamic recognition force mapping. Adhesion force mapping consists in recording a ‘force–volume image’, i.e. an array of force curves in the x, y plane on an area of given size, assessing the unbinding force values for all curves and displaying them as gray pixels [17,74]. The power of this approach in cell surface research is illustrated in Fig. 5 [48].

Topographic images of living mycobacteria immobilized onto porous polymer membranes are shown, together with an adhesion force histogram and adhesion force map (300 nm × 300 nm) recorded with a heparin-modified tip. Adhesion events (clear pixels in the map) were observed in about half of the locations, with a magnitude of ~50 pN, thus very similar to the value of the model HBHA–heparin interaction. This finding strongly suggests that the unbinding forces correspond to the detection of single HBHA, which was further supported by the observation that a mutant strain lacking HBHA did not bind the heparin tip. Interestingly, the HBHA distribution was not homogeneous, but apparently concentrated into nanodomains which may promote adhesion to target cells by inducing the recruitment of receptors within membrane rafts. Other research groups have applied adhesion force mapping to different cell types, including yeast cells [75], red blood cells [66], platelets [76], osteoclasts [77] and endothelial cells [78].

Dynamic recognition force mapping does not provide quantitative force values but is faster and offers better lateral resolution than adhesion force mapping. Here, AFM tips carrying ligands are oscillated at very small amplitudes while being scanned along the biosurface of interest (dynamic force microscopy). Topography and recognition images are simultaneously obtained (TREC imaging) using an electronic circuit [79,80]. Fig. 6 shows how singly distributed avidin molecules can be localized using a biotinylated AFM tip [81]. The nanoscale bright dots visible in the topography image are single avidin
molecules, and the black dots of the recognition image arise from a decrease of the oscillation maxima due to the physical avid–biotin connection during recognition. The lateral positions of the avidin molecules obtained in the topography image are spatially well correlated with the recognition signals of the recognition image (solid circles). In other work, histone H3 in a complex sample of chromatin was recognized by an anti-histone H3 functionalized tip while simultaneously recording a high-resolution image of the same sample [80]. In summary, because the above approaches permit the simultaneous investigation of the topography and of the localization of recognition sites, they should in the future provide valuable insight into the structure–function relationships of biosurfaces.

7. Conclusions

What are the molecular forces driving the interaction of cell adhesion molecules like lectins, integrins, cadherins or...
bacterial adhesins? Does their distribution vary across cell surfaces? These are some of the crucial questions that can now be addressed using single-molecule AFM force spectroscopy. During the past years, rapid advances have occurred in developing reliable, non-destructive procedures for attaching biomolecules and cells on AFM tips (and supporting surfaces). Much progress has also been made in optimizing data acquisition and interpretation in single-molecule force spectroscopy studies, allowing accurate determination of the forces and of the dynamics of a variety of receptor-ligand interactions. In addition, microscopists can use either adhesion force mapping or dynamic recognition force mapping to localize specific binding sites on biosurfaces, thereby providing information that is complementary to that obtained by fluorescence and electron microscopy methods.

In the future, we anticipate that these single-molecule analyses will be increasingly used in biomaterials research for probing cell–cell and cell–material interactions. Combining force mapping with high-resolution imaging on cells will allow to correlate biomolecular interactions with structure. The use of small cantilevers will improve the force resolution, thereby allowing to measure smaller unbinding forces [82]. For imaging, fast speed AFM instruments will enable to improve the time resolution [83,84], therefore allowing to probe dynamic processes that were not accessible before.

Acknowledgments

Y.F.D. is a Research Associate of the FNRS. Our work is supported by the following funds: National Foundation for Scientific Research (FNRS), Foundation for Training in Industrial and Agricultural Research (FRIA), Fonds Spéciaux de Recherche (Université Catholique de Louvain) and Interuniversity Poles of Attraction Programme (Federal Office for Scientific, Technical and Cultural Affairs).

References


