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Immobilization strategies for biological scanning probe microscopy

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Abstract Biological atomic force microscopy (AFM) is now established as a method for studying the structure and function of biomolecular objects at the solid-liquid interface. Major progress in this field is linked to new developments in instrumentation, a better understanding of tip-sample interactions, and improved sample preparation techniques. In this review, the most common strategies for biomolecular immobilization with respect to biological AFM applications are summarized.

Key words: Atomic force microscopy; Scanning probe microscopy

1. Introduction

Scanning tunneling microscopy (STM) and atomic (scanning) force microscopy (AFM) have redefined the concept of microscopy [1,2]. Now standard in the physical sciences, STM and AFM paved the way for the development of a whole family of novel scanning probe microscopes (SPM) with applications extending to biosciences [3,4]. Both techniques are capable of providing topographical information about biomolecular structures that are adsorbed at the solid-liquid interface. AFM, in particular, has attracted the attention of biologists, since it theoretically combines the two most important aspects for studying structure-function relationships of biological objects: high-resolution imaging with high signal-to-noise ratio in the molecular/submolecular range and the ability to operate in aqueous environments, allowing the observation of dynamic molecular events in real-time and under somewhat physiological conditions.

The basic principle in AFM is based on tip-sample force interactions where the surface is raster-scanned in close proximity to a microfabricated tip of atomic scale. The interaction signal is acquired and digitized to provide a three-dimensional image of the surface.

Tremendous progress has been made in the imaging of filamenteous structures, protein arrays and densely packed oligomeric proteins. Lateral resolution in the sub-nanometer range and vertical resolution in the 1–2 Ångström range have been achieved with several biological samples (for reviews see [5–7]). However, lateral resolution is often much lower in the case of single hydrated proteins. This is mostly due to the softness of the biological specimen and the very high pressure in the contact area between tip and specimen. Additional problems are caused by friction effects and lateral displacement which make immobilization strategies critical. Lack of sub-nanometer resolution has driven the community's attention to another benefit of scanning probe microscopy – monitoring the dynamics of biological events in aqueous solution using non-imaging modes. Thus, during the last few years, AFM has evolved from being an imaging device to becoming a method for measuring specific intermolecular recognition forces and intramolecular mechanics [8–10][1].

Other applications take advantage of the atomic-scale positioning capability of the probe for the manipulation of single molecules or other supramolecular structures and organelles [11]. In addition, AFM has been used to mechanostimulate cells and to probe their elastic properties [12–14].

However, AFM of biological macromolecules has yet to become routine in biological laboratories, despite its progress in instrumentation. The major disadvantage of AFM which limits its general use is the fact that lateral resolution is intrinsically dependent on the sample, the finite size and shape of the tip, and the compression due to probe force. For each biological object specific modes have to be adopted. Other problems are the limited understanding of tip-sample interactions and the need to develop SPM-specific preparation methods.

Since the immobilized specimen can deteriorate during preparation and imaging, protocols have to be developed for optimizing the imaging conditions while maintaining the integrity of the immobilized biological structure. Hence, AFM, more than any other imaging technique, requires chemically appropriate and atomically flat substrates to differentiate the topography of adsorbed biomacromolecules, especially single molecules, from that of the solid support. Therefore, the number of suitable substrates is limited because of the combined requirements of flatness and biocompatibility. For this reason, progress in improving AFM-specific protocols will come more from surface science than from other methods in structural biology such as electron microscopy and X-ray crystallography.

In order to probe the structure and/or function of a biological specimen at the solid-liquid interface, immobilization to an atomically flat, texture-free surface is mandatory. The key element is to preserve the activity and integrity of the specimen while firmly anchoring the specimen to the substrate to withstand the lateral forces of the scanning tip. In other words, surface energy, surface charges and hydrophobicity play a critical role. Two different approaches have been used based on the nature of the attachment mode between sample and surface which can be non-covalent or covalent. It is beyond the scope of this minireview to give a comprehensive list of preparation techniques, but I will summarize some of the most popular strategies for molecular immobilization in biological SPM.

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Dedicated to Prof. Giorgio Semenza on the occasion of his 70th birthday.

[1] See also Vinckier and Semenza's review in this issue.

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2. Non-covalent immobilization

The simplest and most commonly used technique for immobilizing a biological specimen is based on physical adsorption (physisorption) from solution where a net attractive force is pulling the specimen onto the solid substrate. A variety of forces are involved in this process including van der Waals (vdW) forces, electrostatic double-layer (EDL) forces, hydration forces and hydrophobic effects [15–17]. The adsorption process is very complex and depends on specimen concentration and purity, charge distribution on the surface of the specimen and the substrate, the counterions involved, the size and polarity of the specimen, and the ionic strength and pH of the buffer. Subsequent events can follow the initial adsorption process such as conformational changes, surface migration, desorption, aggregation, denaturation, chemical reactions, and displacement by the scanning tip. However, the major advantage of this immobilization strategy is its simplicity since it does not require major surface functionalization steps.

The most popular substrate in that respect is muscovite mica, a highly negatively charged, hydrophilic aluminosilicate whose crystals exhibit a large degree of basal cleavage, allowing them to be split into very thin atomically flat sheets with six-fold symmetry. Mica has been successfully used in numberless studies especially for AFM imaging of double stranded DNA and DNA-protein complexes [5,18], protein arrays [17], and densely packed proteins [19,20].

AFM of double stranded DNA has been the subject of great interest and highly reproducible images have been obtained on mica using a variety of protocols. DNA, although negatively charged, has been shown to adsorb firmly to mica and spread out if divalent cations such as Mg^{2+} or Ni^{2+} in HEPES buffers are used as salt bridges between the DNA backbone and the negatively charged mica surface [21–23]. Similar protocols can be used for the deposition of DNA-protein complexes, although in this case the DNA should not be as firmly anchored to guarantee accessibility and activity of interacting proteins.

Within the past years, a series of remarkable high resolution studies of proteins have been published by the groups of Engel and Shao. When proteins are laterally stabilized by close-packing or constrained in a two-dimensional crystal lattice, resolution is subject to reduced contact pressure and less affected by the deteriorating influence of the scanning tip. One of the first high resolution images taken under aqueous conditions was shown by Shao and coworkers with bacterial toxins such as cholera and pertussis toxin B oligomers revealing pentameric subunit structures with 1-nm central openings [19,20]. Other examples include *Escherichia coli* GroES [24], human IgG [25], and smooth muscle myosin [26]. Image acquisition of the last two at 85 K performed with the first cryo-AFM set-ups could further improve resolution, albeit with paying the prize of working on frozen specimen. In all these studies protein purity and very low concentrations were critical parameters to avoid contamination of the scanning tip.

Two-dimensional protein crystals, mainly investigated by Engel and coworkers, have demonstrated the resolving power of the AFM. In some of these cases even conformational changes have been resolved, e.g. force-induced reversible conformational changes of single loops of purple membranes [27], 'open' and 'closed' conformations of OmpF porin trimers with vertical resolution of 1 Å and lateral resolution of 1 nm [28], and time-dependent changes in the inner surface of the hexagonally packed intermediate (HPI) layer from the cell envelope of *Deinococcus radiodurans* [28,29]. The adsorption behavior of these protein arrays on mica has been optimized using an approach that is partly based on the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory [30]. In many cases, these 2D protein crystals adsorb firmly on mica when increasing electrolyte concentrations are used to reduce the repulsive double-layer forces which makes attractive vdW interactions the dominant forces [15–17].

Chemical modification of mica is of importance for extending the number of possible applications. For example, cation exchange has been used to site-specifically immobilize polyarginine tagged green fluorescent protein to the mica surface to improve uniform orientation [31]. Silanization can help to promote the adhesion of biopolymers. For example, two-dimensional organosilane polymers formed by condensation of 3-aminopropyl-triethoxysilane (APTES) adsorb readily to mica and reverse the charge through their protonated amino groups at neutral pH [32]. These surfaces have been successfully used as substrates for dsDNA and ssRNA adsorption [22,33–35]. Although not necessary for organosilane formation and adsorption, the density of surface silanol groups can be increased by means of plasma discharge in water vapor without significant increase in surface roughness [36].

Hydrophobic surfaces are usually not recommended as substrates for solution-based measurements. Beside the fact that they are not wettable, they often distort AFM imaging due to increased adhesion forces and they tend to denature proteins. In some cases, however, hydrophobic surfaces can be a valuable alternative. For example, hydrophobic silanized glass surfaces have been used to adsorb specifically the inner surface of HPI layers in order to facilitate AFM imaging of the hydrophilic outer surface [37]. Carbon-coated mica has been used to create a hydrophobic flat substrate for DNA imaging [38].

Cationic-supported lipid bilayers are another promising substrate for physical adsorption of DNA as introduced by Shao's group. DNA adsorbs firmly to these positively charged bilayer surfaces when divalent cations are removed by EDTA treatment. The resulting high-resolution images taken in solution revealed even the double helix pitch of 3–4 nm and its right-handedness [39].

Physical adsorption has also been used to modify the tip surface for non-topological applications such as chemical mapping and the quantification of inter- and intramolecular forces. Biotinylated BSA has been shown to readily adsorb to the tip surface, making it possible to determine the interaction forces between biotin and avidin [40]. In subsequent studies Rief et al. [9] used the same strategy to investigate the mechanical properties of individual dextran filaments that are anchored at one end to the tip through a biotin-streptavidin bond and at the other end to a gold surface via thiolate linkage. In a second study stretching of recombinant titin molecules was achieved by non-specific adsorption of a fraction of the protein to the tip. Unfolding and refolding of individual immunoglobulin domains were observed [10].

3. Covalent immobilization

In many cases single molecules and filamentous structures

require immobilization where a stable covalent bond is formed between chemical groups of the specimen and functionalities that are exposed at the substrate surface. Covalent immobilization is an important strategy for those applications where displacement or desorption is a critical issue, but also when conditions for adsorption and biological activity are incompatible, or when the molecular objects have to be integrated in complex supramolecular assemblies that include self-assembly processes and require well-defined coupling steps.

With respect to SPM applications, three aspects are critical for covalent immobilization strategies: (i) the immobilization chemistry must be very reproducible, (ii) the integrity and activity of the biomolecule should not be affected by the chemical reaction with the substrate, and (iii) the surface must be atomically flat, hydrophilic, preferentially monofunctional, and should not negatively interfere with the scanning probe. This set of requirements limits the choice of materials considerably.

Only in very few cases biomolecules were directly coupled to an inorganic substrate, such as thiol-modified DNA on gold [41] and titin on gold via cysteine-thiols [10]. In most cases immobilization of biomolecules on chemisorbed ω-functionalized monolayers provides better alternatives to meet the aforementioned requirements. To date mainly two systems have been used for covalent immobilization: alkylsiloxane monolayers on hydroxylated surfaces and alkylthiol monolayers on noble metals. In both cases a high density of reactive functionalities results and if the monolayers are deposited correctly they mirror the flatness of the underlying substrate. When more simple functionalities are chosen (such as hydroxyl-, amino-, and carboxylic acid groups), subsequent activation steps are mandatory. Dozens of synthetic pathways can be conceived for in situ activation chemistry – the reader is referred to the reviews of Wong [42], Taylor [43], and Hermanson et al. [44]. The major disadvantage of this strategy is that multiple functionalization steps and surface analyses are mandatory which is the primary reason that covalent immobilization is not commonly used.

One of the first immobilization protocols on derivatized glass was presented by Karrasch et al. [37]. In this study APTES-treated glass was activated in situ with *N*-5-azido-2-nitrobenzoyl-oxysuccinimide to expose photoreactive headgroups for non-specific photochemical immobilization of proteins. This protocol was successfully used for AFM imaging of proteins, despite the fact that the resulting surfaces are very hydrophobic [37]. In another method, amino-derivatized glass has been activated with glutaraldehyde to immobilize microtubules via their native amino groups [45]. The major disadvantages of alkylsiloxane-derivatized surfaces are the lack of reproducibility in monolayer formation, the need for extensive precleaning steps, and the limited range of chemically compatible ω-functionalities.

The second system for preparing atomically flat monolayers is based on the spontaneous assembly of alkylthiols and dialkyldisulfides on gold surfaces. The resulting close-packed monolayers can easily be prepared from liquid or vapor phase with very high reproducibility and display a wide variety of functional groups [46,47]. Since SPM applications require extremely flat surfaces, a method was developed to prepare atomically flat gold surfaces for these monolayer systems [48,49]. These template-stripped Au(111) surfaces are prepared by vapor-deposition of gold on hot mica with subsequent removal of the mica template to use the first gold atom layer that is deposited on the mica resulting in roughness values of a few Ångström per hundreds of μm^2. The chemical and physical properties of a variety of ω-functionalized monolayers have been studied on these flat gold surfaces, one of them with *N*-hydroxysuccinimide headgroup functionality is a particular well-studied system [50]. This amino-reactive monolayer has been used for tethering amino-modified dsDNA molecules specifically at their ends, an important asset for DNA-protein interaction studies. In addition, it has also shown general applicability for immobilizing a variety of proteins including clathrin where the disassembly of immobilized cages could be followed in situ by AFM [51,52]. The very same immobilization system has been employed for non-imaging applications using functionalized AFM tips. Covalent immobilization of proteins onto the very end of gold-coated tips made it possible to determine the interaction forces between single proteoglycan molecules [53] and, in a second study, the forces intrinsic to antigen-antibody binding [54]. Other applications based on alkylthiol-modified probes include chemical sensing and the determination of adhesion and friction effects on chemically patterned surfaces [55].

4. Future perspectives

Within the next years, progress in scanning probe microscopy will greatly benefit from new technical developments with new modes of operation, hybrid instruments, refined preparation protocols, and especially with a new generation of probes. Well-defined chemical modification of probes will enable new applications in chemical and biological sensing, partly based on novel micromechanical cantilever designs capable of detecting phenomena such as surface stress [56]. Highly flexible, multiwalled carbon nanotubes attached to conventional AFM tips might improve imaging conditions and resolution and work as high aspect-ratio chemical probes if functionalized [57]. Arrays of cantilevers are currently in development at Stanford and other places and will help to reduce data acquisition times. New immobilization strategies and novel substrates, such as atomically flat ω-functionalized monolayers on oxide-free Si(111) will be further optimized and applied to biological samples [58]. All these applications require chemical and biochemical functionalization and the choice of substrate and immobilization chemistry remains a critical part.

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