

## INVITED REVIEW

# Sample preparation procedures for biological atomic force microscopy

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## Summary

Since the late 1980s, atomic force microscopy (AFM) has been increasingly used in biological sciences and it is now established as a versatile tool to address the structure, properties and functions of biological specimens. AFM is unique in that it provides three-dimensional images of biological structures, including biomolecules, lipid films, 2D protein crystals and cells, under physiological conditions and with unprecedented resolution. A crucial prerequisite for successful, reliable biological AFM is that the samples need to be well attached to a solid substrate using appropriate, nondestructive methods. In this review, we discuss common techniques for immobilizing biological specimens for AFM studies.

## 1. Introduction

During the past two decades, the atomic force microscope (AFM) (Binnig *et al.*, 1986) has provided new avenues for microscopists to study biological specimens. AFM yields three-dimensional images of biosystems (single molecules adsorbed on surfaces, lipid membranes, 2D protein crystals, living cells) in aqueous solutions and with (sub)nanometre resolution. In addition, it can also measure forces with remarkable sensitivity and positional precision. In this mode, known as force spectroscopy, the cantilever deflection is recorded as the tip is pushed towards the sample and retracted from it. Force spectroscopy can be used to probe quantitatively physical properties such as local elasticity, surface forces, surface charge and hydrophobicity and to measure inter- and intramolecular interactions, providing new insights into the molecular bases of processes such as protein folding and receptor–ligand interactions (for an overview of imaging and force spectroscopy applications, see Jena & Hörber, 2002).

In this paper, we provide a survey of the various methods used for preparing samples for biological AFM. We first describe how to select and prepare appropriate substrates and then discuss immobilization protocols available for isolated molecules, supported lipid films, two-dimensional protein crystals and cells. Although not the primary goal of this review, we also briefly highlight some important imaging applications in biosciences (for detailed information on methodologies and applications, see Ikai, 1996; Shao *et al.*, 1996; Colton *et al.*, 1998; Morris *et al.*, 1999; Jena & Hörber, 2002).

## 2. Substrates

After the first AFM images of biological structures were obtained, it was soon realized that a crucial factor for successful imaging is the specimen preparation. The first issue is to select an appropriate substrate. Indeed, to observe biological structures in their native state, these must be well attached to a smooth solid substrate to resist the lateral forces exerted by the scanning tip. In that respect, mica, glass and silicon oxide have proved to be excellent substrates. Muscovite mica,  $\text{KAl}_2(\text{OH})_2\text{AlSi}_3\text{O}_{10}$ , is a nonconducting layered mineral composed of multiple 1 nm thick layers (Bailey, 1984). It can be cleaved easily with the help of adhesive tape to produce clean, atomically flat surfaces that are negatively charged. Today, mica is the most frequently used substrate for imaging biological specimens, including double-stranded DNA, DNA–protein complexes, protein arrays, densely packed proteins, supported lipid films and animal cells. Interestingly, the mica surface can be modified with silanes either to promote adsorption or to allow covalent binding of the biomolecules (Lyubchenko *et al.*, 1992).

Glass represents another suitable substrate for biological AFM. Glass cover slips are flat enough for imaging cells and other large structures, but are usually too rough for imaging adsorbed molecules. The glass surface is always coated with organic contaminants and particles that should be removed before use, e.g. by washing in concentrated acidic solution

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followed by ultrasonication in water solutions. As for mica, glass can also be modified with silane molecules, bringing new chemical functions at the surface for further covalent modifications (Karrasch *et al.*, 1993). In some cases, silicon oxide wafers initially developed for the semiconductor industry can be used instead of glass. Although they are more expensive and more difficult to handle, they have a much smoother surface than glass.

Hydrophobic substrates may sometimes be preferred. A well-known example is highly orientated pyrolytic graphite (HOPG), which is atomically flat over large areas (Cullen & Lowe, 1994). Coating the mica surface with carbon yields a hydrophobic surface that is well suited for immobilizing DNA (Yang *et al.*, 1992). Spin-coating of polymers [polystyrene, poly(methyl methacrylate)] is another convenient method for creating flat hydrophobic substrates for biological AFM (Dupont-Gillain *et al.*, 1999). For some applications requiring specific surface chemistries, gold surfaces may be useful. Thin layers of gold can be prepared by thermal evaporation onto mica, glass or silicon oxide substrates. To obtain very smooth gold surfaces (roughness  $\sim 0.1$  nm), various methods, referred to as template stripped gold (TSG) methods, have been developed (Wagner *et al.*, 1995). The typical procedure involves depositing gold onto a smooth substrate such as silicon, supporting the free gold surface by gluing it to a glass slide using epoxy glue and stripping the gold film from the silicon substrate. The advantage of using gold is that it is chemically inert against oxygen and stable against radicals and can be easily modified with self-assembled monolayers (SAMs) of organic alkanethiols that can be used further for adsorbing or attaching biomolecules. Finally, as we shall see below, three-dimensional systems such as porous polymer membranes and macromolecular gels can sometimes be used to immobilize large objects such as cells.

### 3. Biomolecules

Several approaches may be used for preparing biomolecules for AFM in air. A simple immobilization procedure consists of depositing a drop of an aqueous solution containing the macromolecule of interest on the substrate, and then allowing the drop to evaporate or blowing the sample dry in nitrogen. Alternatively, the substrate can be immersed in the solution to allow adsorption of the biomolecules for a given period of time, rinsed and then air-dried. Finally, the molecules can also be sprayed with an aqueous solution, or in the presence of glycerol, onto the substrate. DNA alone or in association with proteins has been deposited using these protocols, and then imaged in air (Vesenska *et al.*, 1992; Rees *et al.*, 1993), under alcohols (Hansma *et al.*, 1992) or in aqueous solution (Bezanilla *et al.*, 1993). Similar protocols have been applied to proteins such as fibronectin (Emch *et al.*, 1992) or collagen (Chernoff & Chernoff, 1992), and to polysaccharides (Kirby *et al.*, 1996).

Physical adsorption in the presence of appropriate electrolytes is another approach which offers the advantage of allowing

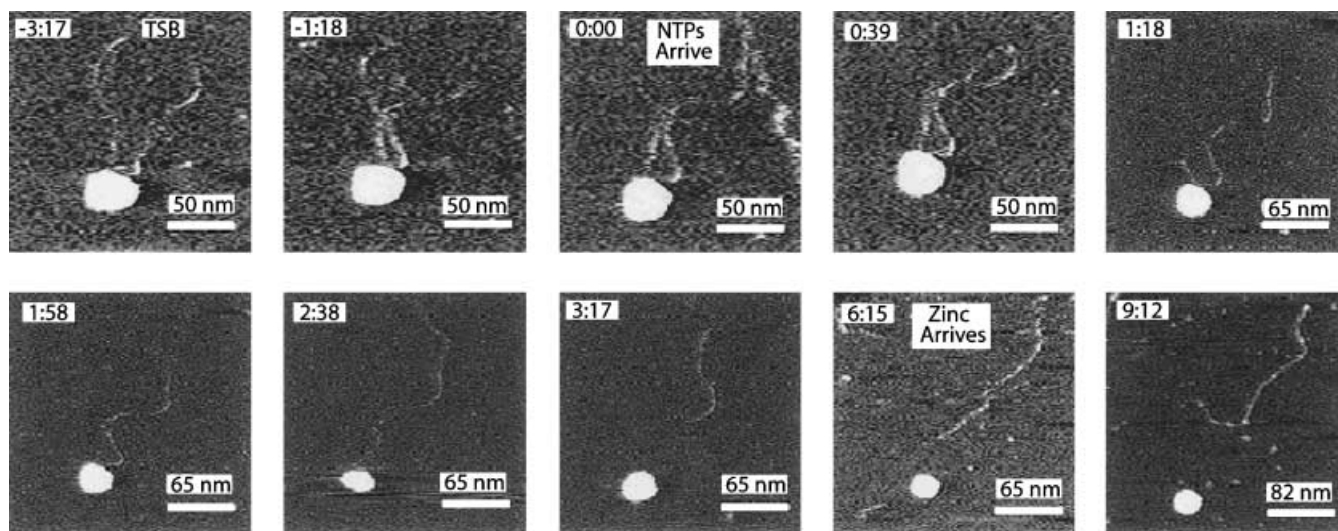
the sample to be directly imaged under liquid without the need for air-drying. A wide range of biomolecules, primarily proteins and DNA, have been imaged by AFM while being adsorbed on mica (Hoh *et al.*, 1993; Radmacher *et al.*, 1994; Yang *et al.*, 1994; Fritz *et al.*, 1995; Hansma *et al.*, 1995). For soluble proteins, the very first *in situ* images were generally of poor resolution (Lin *et al.*, 1990). However, since then there has been continuous progress in improving preparation methods and imaging conditions for these specimens (for reviews, see, e.g. Shao *et al.*, 1996; Wagner, 1998). For instance, in an early high-resolution study, pertussis toxin could be imaged down to a resolution of 0.5 nm following simple adsorption on mica (Yang *et al.*, 1994). As discussed below (Section 5), proteins that form stable two-dimensional crystals are actually very well-suited for high resolution imaging.

Various approaches may be used to promote the attachment of adsorbed biomolecules. For DNA, pretreating the substrate with silanes bearing an amino group such as 3-aminopropyltriethoxysilane favours attachment (Lyubchenko *et al.*, 1992). DNA molecules were also found to bind tightly enough on mica when the solution contains 1 mM concentrations of certain divalent cations, i.e.  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  or  $\text{Mn}^{2+}$  (Hansma & Laney, 1996). Interestingly, by adjusting the concentration of  $\text{Zn}^{2+}$ , it is possible to bind DNA sufficiently so that it can be imaged but loosely enough to be translocated by the RNA polymerase (Kasas *et al.*, 1997; Fig. 1). For proteins (lysozyme, streptavidin, the cholera toxin B-subunit, GroES and GroEL), it has recently been shown that monovalent cations ( $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+$ ) can inhibit the adsorption to mica, suggesting that reducing this inhibition may be a generally useful procedure to maximize the amount of proteins on this surface (Czajkowsky & Shao, 2003).

Covalent attachment of macromolecules may sometimes be desired. Immobilization of proteins on glass may be achieved using a photoreactive cross-linker (Karrasch *et al.*, 1993; see Section 5). Another elegant approach is to form *N*-hydroxysuccinimide-terminated monolayers on gold-coated substrates, using, e.g. dithio-*bis*-succinimidylundecanoate, and then binding the proteins covalently in aqueous buffers under mild conditions (Wagner *et al.*, 1996; Wagner, 1998). Finally, it is worth noting that covalent binding of molecules on AFM tips and/or substrates is also used for measuring specific biomolecular interactions by force spectroscopy. Here, several flexible spacer molecules have been introduced, including poly(ethylene glycol) (Hinterdorfer *et al.*, 1996) and carboxymethyl-amylose (Grandbois *et al.*, 2000), in order to provide enough mobility to the attached biomolecules so that they can freely interact with complementary molecules.

### 4. Supported lipid films

Thin lipid films supported on solid substrates are valuable model systems for mimicking biological surfaces. In particular, these systems are widely used in biophysical research to investigate



**Fig. 1.** Adsorption in the presence of appropriate ions allows to observe biomolecules at work. In this example, immobilization of linear double-stranded DNA templates on mica in the presence of  $\text{Zn}^{2+}$  allowed imaging of transcription by *Escherichia coli* RNA polymerase (RNAP). The transcription process was detected by observing the translocation of the DNA template by RNAP on addition of ribonucleoside 5'-triphosphates (NTPs) in sequential AFM images. The first two images show that before the NTPs enter, the DNA has mobility on the mica. The six images after NTP addition, from time 0:00 onwards are sequential and show that one arm of the DNA template becomes progressively shorter until it is released (02:38). Reprinted with permission from Kasas *et al.* (1997). © 1997, American Chemical Society.

the properties of biological membranes and processes such as molecular recognition, enzymatic catalysis, cell adhesion and membrane fusion (McConnell *et al.*, 1986; Sackmann, 1996). Transferring lipid films onto solid substrata offers the possibility of applying a range of surface analytical techniques, and more particularly AFM.

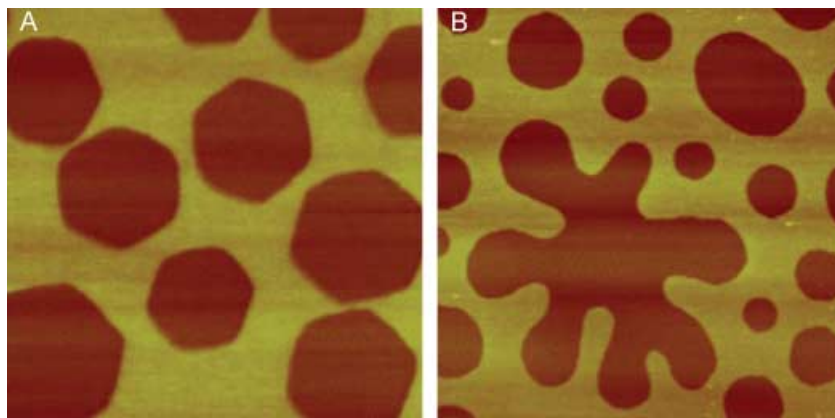
For AFM imaging applications, supported lipid films may be prepared either by the Langmuir–Blodgett (LB) technique or by fusion of lipid vesicles (Czajkowsky & Shao, 2002). In the first method, a Langmuir trough consisting of a rectangular Teflon bath equipped with moveable barriers is used to compress the lipid molecules at the air–water interface (Ulman, 1991). Lipids are usually spread at the air–water interface in hexane/ethanol or chloroform/methanol mixtures, and then compressed after letting the solvent to evaporate for 15 min. A sensor records the surface pressure at the interface, which can be expressed as a function of the interfacial area. The obtained surface pressure vs. area isotherms can provide useful information on the packing and organization of the lipid molecules. In the LB technique, the monolayer of amphiphilic molecules is transferred at constant surface pressure and constant speed onto a solid substrate, usually mica. Careful control of surface pressure and lifting speed is essential to avoid artefacts such as defect formation or feature alignment of the deposited structures. Lipid monolayers interact with mica through the polar heads, thus exposing the hydrophobic tails to the environment. These systems are stable in air, not in water, and should therefore be examined in air by AFM. Transferring a second lipid layer onto a mica-supported lipid monolayer yields a supported bilayer, which best mimics cellular membranes. These

supported bilayers should always be kept and analysed in aqueous solution, as they are not stable in air.

Fusion of lipid vesicles on solid substrates is another approach to obtain supported lipid bilayers (Brian & McConnell, 1984; Horn, 1984). Typically, the lipids are first solubilized in organic solvent. After solvent evaporation under nitrogen and subsequent desiccation under vacuum, the dried lipid film is resuspended in aqueous buffer solution (usually Tris or PBS) yielding a multilamellar vesicles (MLVs) suspension. From this suspension, small unilamellar vesicles (SUVs) can be obtained using various approaches, sonication being the most popular one. The suspension is sonicated to clarity (e.g. five cycles of 2 min) using a titanium probe sonicator while keeping the suspension in an ice bath, after which the suspension is filtered on 200 nm nylon filters to eliminate titanium particles. Then fusion is achieved by heating the SUV suspension in contact with freshly cleaved mica for 1 h at a temperature between 45 °C and 60 °C. The supported bilayers are finally gently cooled to room temperature and rinsed abundantly with the appropriate imaging buffer. Compared to LB deposition, the drawbacks of the fusion method are the impossibility of preparing asymmetric bilayers composed of two layers of different nature as can be easily obtained with the LB technique, and the lack of control of the lateral pressure in the lipid layers. However, because the fusion approach is much simpler and permits lipid diffusion as in free-standing bilayers, it is the most widely used method in AFM lipid bilayer research.

Since the first AFM images revealing the molecular structure of supported phospholipid films were published (Egger *et al.*, 1990), a broad spectrum of AFM applications has emerged in





**Fig. 2.** Imaging supported lipid monolayers prepared by the Langmuir–Blodgett (LB) technique. AFM friction images in air of mixed fengycin/ceramide monolayers supported on mica prepared at 20 °C/pH 2 (A; 2  $\mu\text{m} \times 2 \mu\text{m}$ ) and 37 °C/pH 5 (B; 15  $\mu\text{m} \times 15 \mu\text{m}$ ). The images reveal phase-separation in the form of two-dimensional hexagonal or circular domains of ceramide surrounded by a fengycin-enriched fluid phase and demonstrate the dramatic influence of the environmental conditions (i.e. temperature, pH) on the molecular organization of the films. Reprinted with permission from Eeman *et al.* (2005). © 2005, American Chemical Society.

lipid film research (for reviews see, e.g. Dufrêne & Lee, 2000; Czajkowsky & Shao, 2002), among which are visualizing the molecular structure and organization of phase-separated films (Dufrêne *et al.*, 1997; Vié *et al.*, 1998; Eeman *et al.*, 2005; Fig. 2), probing the effect of external agents such as solvents (Mou *et al.*, 1994a, 1994b), peptides (Rinia *et al.*, 2002; El Kirat *et al.*, 2005), proteins (Milhiet *et al.*, 2002) and antibiotics (Berquand *et al.*, 2004; Fig. 3) and using lipid films as substrates for anchoring biomolecules such as DNA (Mou *et al.*, 1995a) and crystalline protein arrays (Reviakine *et al.*, 1998).

## 5. Two-dimensional protein crystals

Owing to their stability and structural regularity, two dimensional (2D) bacterial protein crystals (Sleytr & Beveridge, 1999) have proved to be excellent systems for high resolution AFM imaging. AFM yields structural information with a lateral resolution better than 1 nm, directly under physiological conditions, which makes it a complementary tool to X-ray and electron crystallography (for an overview of applications in the field, see Müller & Engel, 2002).

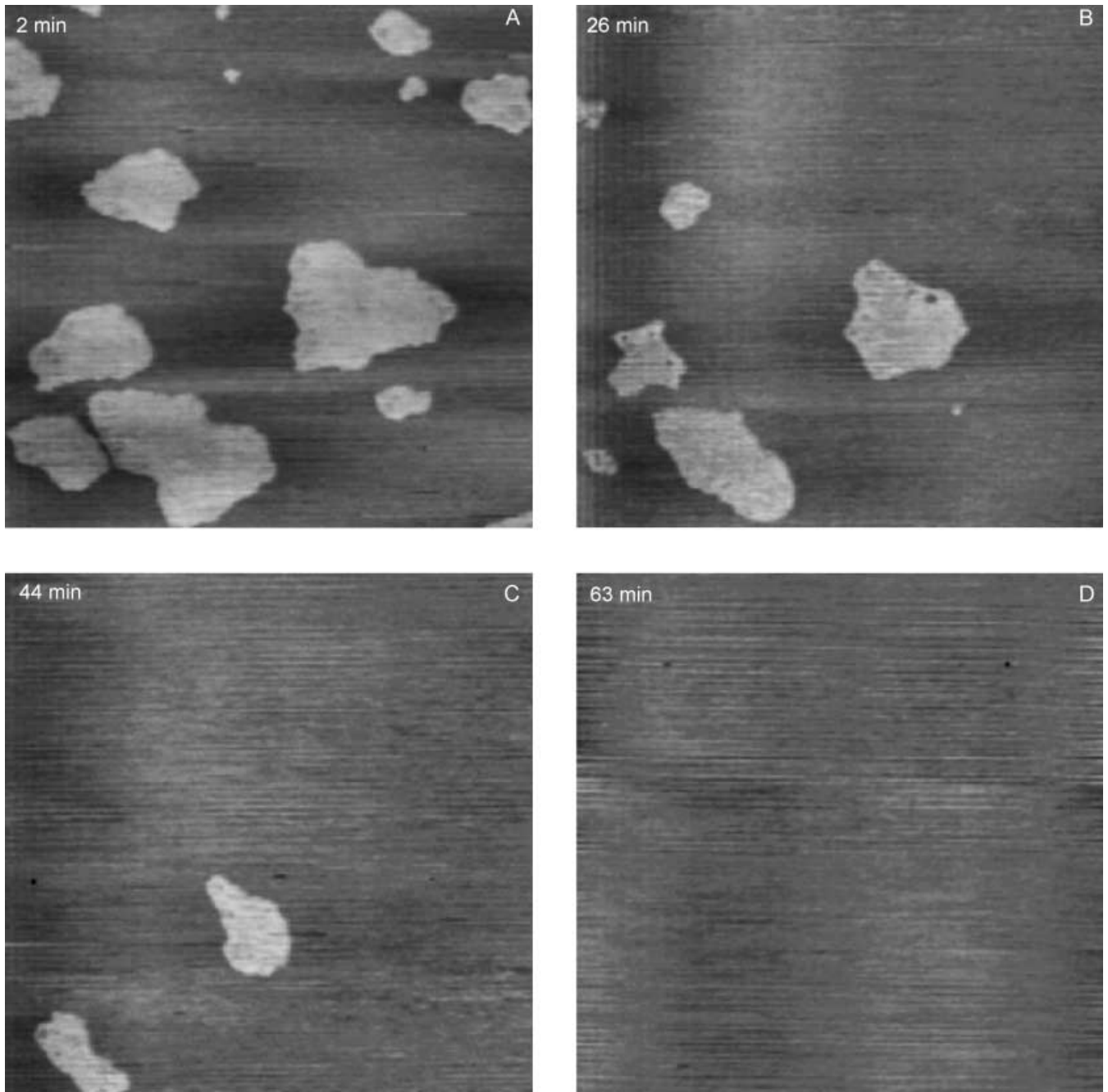
Several immobilization strategies have been established for 2D protein crystals, the most frequently used procedures being based on physical adsorption in aqueous solution (for reviews, see Müller *et al.*, 1997a,b). A well-known example is the purple membrane from *Halobacterium salinarum*, which readily adsorbs on mica. The typical procedure for adsorption is as follows (Müller *et al.*, 1997a): 150 mM KCl is added with 10 mM Mes, Hepes or Tris for adjusting the pH; the purple membranes are diluted in the buffer to a concentration of about 50  $\mu\text{g mL}^{-1}$  and a drop of this solution is deposited onto freshly cleaved mica; after adsorption for 10–30 min, samples are gently washed with buffer to remove membranes that are not firmly attached. Using this procedure, individual bacteriorhodopsin molecules in the membrane can be imaged at subnanometre resolution (Fig. 4). Furthermore, force-induced conformational changes can be directly visualized.

Another example is the channel-forming protein OmpF porin from *Escherichia coli* that forms 2D crystals when reconstituted

in the presence of lipids. Immobilization onto freshly cleaved mica is achieved in the presence of small amounts of divalent ions (2 mM  $\text{MgCl}_2$ ) and monovalent ions (NaCl) between 100 and 150 mM (Schabert & Engel, 1994; Schabert *et al.*, 1995). The presence of  $\text{Mg}^{2+}$  is essential to compensate the negative surface charges of both sample and substrate. This protocol allowed high-resolution imaging of porin OmpF 2D crystals, with a lateral resolution of 1 nm and a vertical resolution of 0.1 nm (Schabert *et al.*, 1995).

To enhance the adsorption of negatively charged samples, the surface properties of the substrate can be modified by coating with a polycation such as poly L-lysine (Müller *et al.*, 1997b). Crosslinkers can also be used to covalently anchor membrane proteins on substrates. An original procedure has been developed for the hexagonally packed intermediate (HPI) layer of *Deinococcus radiodurans*. It involves the chemical modification of glass with the photoactivatable cross-linker N-5-azido-2-nitrobenzoyloxysuccinimide, followed by cross-linking the samples by irradiation with ultraviolet light (Karrasch *et al.*, 1993; Karrasch & Engel, 1998). This procedure allowed the HPI layer proteins to be imaged in buffer solution with a lateral resolution of 1 nm. Covalent immobilization has the disadvantage of requiring more preparation steps and may alter the native structure of the biological specimen.

Lipid monolayers and bilayers have also proved to be a valuable preparation method for immobilizing recrystallized protein arrays. A first approach, used for instance for *Bacillus* S-layers, consists in recrystallizing the proteins on a lipid monolayer in a LB trough and then transferring the composite structure on a flat substrate for AFM examination (Pum *et al.*, 1993). Alternatively, supported lipid bilayers containing various ligand-linked lipids may be used to specifically bind the proteins. Examples of such protein crystallization procedures have been reported for streptavidin (Weisenhorn *et al.*, 1992; Scheuring *et al.*, 1999), cholera toxin (Mou *et al.*, 1995b) and annexin V (Reviakine *et al.*, 1998), which, respectively, bind to biotinylated lipids, GM1 ganglioside and negatively-charged lipids such as phosphatidylserine.

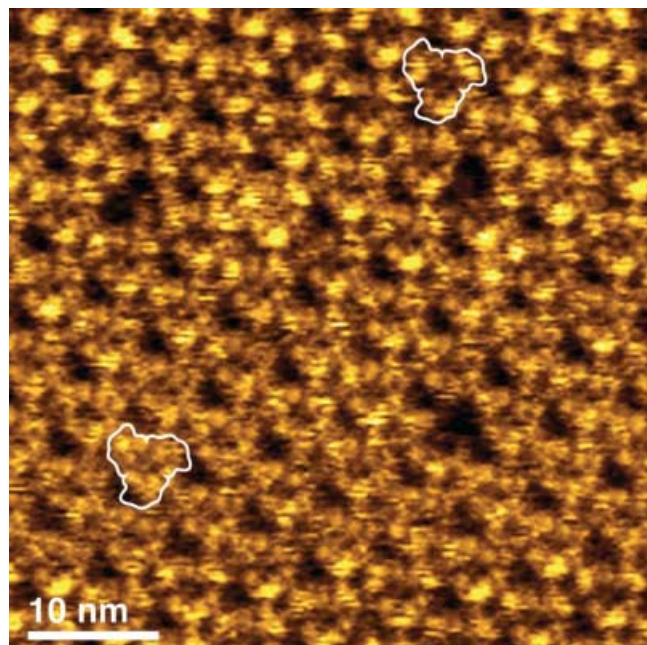


**Fig. 3.** Real-time imaging of supported lipid bilayers prepared by vesicle fusion. AFM height images ( $7.5\ \mu\text{m} \times 7.5\ \mu\text{m}$ ; z-scale: 10 nm) of a mixed dioleoyl-phosphatidylcholine (DOPC)/dipalmitoyl-phosphatidylcholine (DPPC) bilayer recorded in solution at increasing incubation times with the antibiotic azithromycin. The antibiotic causes progressive erosion of the gel DPPC domains, an effect attributed to the intercalation of the drug at the domain boundary. Reprinted with permission from Berquand *et al.* (2004). © 2004, Elsevier Science Ltd.

## 6. Cells

The potential of AFM for probing cells was recognized quite early (Butt *et al.*, 1990). Here, a key challenge has been to develop procedures allowing imaging of the cells in their native state. For animal cells, a simple preparation method is to exploit the ability

of the cells to spread and adhere to solid surfaces (Radmacher *et al.*, 1992; Matzke *et al.*, 2001). Coating the substrate with adhesion proteins such as collagen may be used to enhance immobilization, a procedure which allowed the observation of actin filament dynamics under the cell membrane of glial cells (Henderson *et al.*, 1992). A rather different approach is to image



**Fig. 4.** Adsorption of 2-D protein crystals on mica, here the purple membrane, allows to record high-resolution AFM images of their surface in aqueous solution. A fully reversible force-induced conformational change is observed: at the top of the image the force applied to the AFM stylus is 100 pN; while scanning the surface line by line the force is increased until it reaches 150 pN at the bottom of the image. Reprinted with permission from Müller *et al.* (2000). © 2000, Elsevier Science Ltd.

living cells fixed only by a suction pipette, using an AFM combined with an optical microscope (Hörber *et al.*, 1992). Cells can be kept alive for days in growth medium while being examined, making it possible to study cell activities and dynamics. Finally, in some cases chemical fixation of the cells 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 (Le Grimmelc *et al.*, 2002). Owing to these different preparation methods, various cell types have been investigated, such as CV-1 kidney cells, fibroblasts, MDCK, platelets and cardiomyocytes (Jena & Hörber, 2002). An important achievement in cell biology has been the possibility of following dynamic processes at cell surfaces, as was shown for instance for the plasma membrane of pancreatic acinar cells, where depressions attributed to fusion pores could be observed (Schneider *et al.*, 1997).

As opposed to animal cells, microbial cells (bacteria, yeast and fungi) cannot spread over solid substrates, meaning immobilization by means of simple adsorption procedures is often inappropriate, as this most often leads to cell detachment by the scanning probe. Stronger attachment may be achieved either by pretreating the substrate with polycations (Schaer-Zammaretti & Ubbink, 2003) or by bonding the cells covalently to the substrate (Camesano *et al.*, 2000). The ability of microorganisms to attach to solid substrates by means of extracellular polymeric substances may also be exploited for AFM studies, but the

resolution remains limited (Beech *et al.*, 1996). Another possibility is to immobilize the cells mechanically 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 (Gad & Ikai, 1995). 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 (Kasas & Ikai, 1995; Dufrêne *et al.*, 1999; Fig. 5). In a recent comparative study, it was concluded that, in general, mechanical trapping of bacterial cells in filters appears to be the most reliable method for immobilization (Vadillo-Rodriguez *et al.*, 2004). Physical adsorption on modified substrata was suggested to promote structural rearrangements in bacterial cell surface structures, whereas glutaraldehyde treatment was shown to induce changes on cell surface properties.

Another advantage of the mechanical trapping approach is that it allows observing dynamic processes. In one such study, Touhami *et al.* (2004) combined AFM imaging in aqueous solution and thin-section transmission electron microscopy (TEM) to investigate the changes in the cell wall of *Staphylococcus aureus* cells as they grow and divide. A good correlation of the structural events of division was found using the two techniques and the AFM was shown to provide new information.

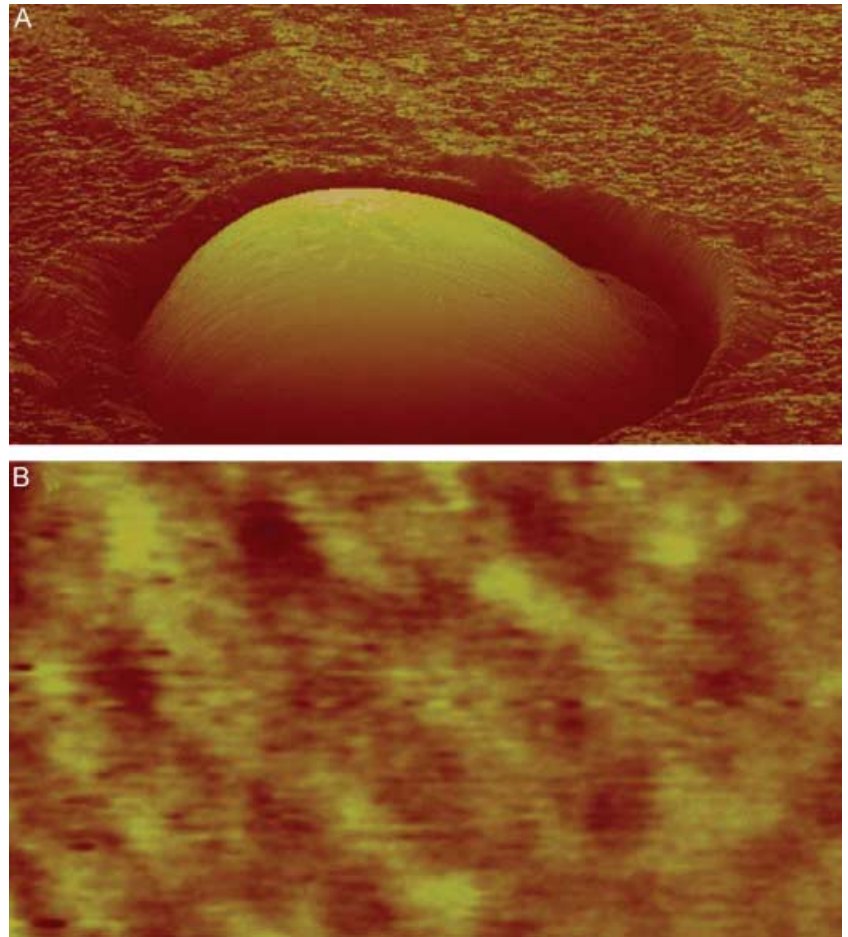
## 7. Perspectives

Since the birth of AFM, tremendous advances have been made in developing sample preparation methods for biological specimens. Today, simple, reliable protocols are available for most specimens, meaning that, in general, sample preparation is no longer a bottleneck for biological AFM. However, there are still many technical challenges to address in the field. In particular, high-resolution imaging on soft samples such as animal cells remains often very difficult due to the damaging effects of the scanning tip. To solve this issue, several approaches are being developed (Hörber & Miles, 2003). For instance, tip-specimen interaction forces can be greatly reduced using tapping mode AFM with an active resonance control (Tamayo *et al.*, 2001). Another promising approach is the photonic force microscope, in which the AFM cantilever is replaced by the 3D trapping potential of a laser focus, allowing 3D imaging of biological structures with extremely small loading forces (Pralle *et al.*, 2000). Time resolution is another problem in current biological AFM studies, as 30–60 s are usually needed to record an image. In this context, new ultra-fast instruments provide exciting avenues to explore dynamic processes associated with biomolecules and cells (Viani *et al.*, 2000; Ando *et al.*, 2001; Hörber & Miles, 2003).

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**Fig. 5.** Imaging living microbial cells immobilized in porous polymer membranes. Low (A;  $3\ \mu\text{m} \times 1.5\ \mu\text{m}$ ) and high (B;  $500\ \text{nm} \times 285\ \text{nm}$ ) resolution AFM images obtained for the bacterium *Lactococcus lactis*. The cell surface is quite smooth in agreement with the presence of peptidoglycan, which is the major determinant of cell shape and rigidity.

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