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Immobilization of microorganisms for AFM studies in liquids

Tobias J. Günther[1*], *Matthias Suhr*[2*], *Johannes Raff*[1,2], *Katrin Pollmann*[1]

[1] Helmholtz-Zentrum Dresden-Rossendorf, Helmholtz Institute Freiberg for Resource Technology, Bautzner Landstraße 400, 01328 Dresden, Germany

[2] Helmholtz-Zentrum Dresden-Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, 01328 Dresden, Germany

*Both authors have equally contributed

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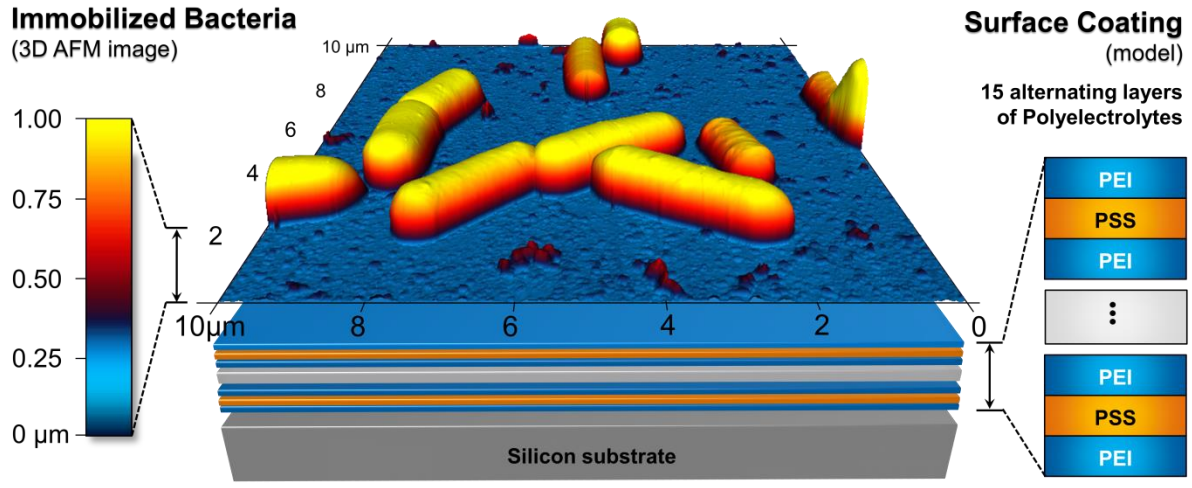
"**Reproducible immobilization method even for living eukaryotes and prokaryotes on polyelectrolyte coated surfaces for high resolution AFM imaging in liquids.**"



Atomic force microscopy image of immobilized bacteria (*Lysinibacillus sphaericus* JG-B53) on polyelectrolyte supported surface and model of layer constitution of new immobilization matrix.

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ABSTRACT. In this paper a new sample preparation method is described that allows for the *in-vivo* AFM imaging of a wide range of different microorganisms. The primary focus of this work was on the immobilization of fixed and living cells of various microorganisms on substrates. The tested organisms of interest were Gram-negative and Gram-positive bacteria, yeast, and algae. The immobilization of the biological samples on a sample holder is crucial for AFM. Lateral forces of the probe tip can alter or remove sample material during scanning. This effect occurs especially on soft biological samples, which causes artifacts within the imaging and leads to a loss in quality and structural information. For the immobilization organisms were deposited on polyelectrolyte coated surfaces by centrifugation. Microorganisms were imaged without the use of any drying steps including either living or with glutaraldehyde fixation. Glutaraldehyde fixation enables long time scans that cover wide areas or the investigation of organisms in special growth stages, such as cell division or budding. Skipping fixation steps allows *in vivo* imaging to investigate living organisms and cellular processes under physiological conditions. A method for the reliable and efficient immobilization of microorganisms has been demonstrated by imaging the proteinaceous surface layer (S-layer) of living *Lysinibacillus sphaericus* and *Viridibacilli arvi* cells. In additional experiments, cell division of *E. coli* was successfully imaged. During repeated wide area scans, fixed sample material was not removed by the AFM tip, proving the suitability of these methods for AFM analyses. Ultimately, this method can be easily applied for the immobilization of a wide range of microorganisms and *in vivo* imaging of whole cells and cell ultrastructure.

INTRODUCTION. Since the invention of AFM (atomic force microscopy) in 1986 by Binning and colleagues[1], AFM has become a versatile tool not only for material scientists, but also for biologists and chemists. An atomic force microscope has several modes of operation, mainly contact or non-contact modes for imaging and force spectroscopy for material characterization. It is commonly used for imaging surface topography at the nanometer scale, but also operates as a nano-manipulating device that allows for the characterization of surfaces and even single molecules. In the past years AFM was used in the field of biology for the high resolution imaging and manipulation of specimen in liquids under physiological conditions[2-5]. AFM was used to gain high-resolution images of prokaryotic cells [6, 7], eukaryotic cells[8], water sol-

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uble proteins[9, 10], self-assembling and tailor-made proteins [11, 12], DNA-protein complexes[13-15], and lipid bilayer[16] and membrane proteins[17-19]. In addition to imaging, the field of application includes mechanical investigations like adhesion measurements[20, 21], single molecule force spectroscopy [22-24], cell mechanical measurements[25, 26], and even the unfolding of proteins[22, 27]. Nanolithography is another operating mode used for biochemical surface modification[28]. In the past AFM has been proven to be a versatile tool for high resolution imaging of structures in the nanometer range. One example of this is the *in vitro* imaging of S-layer sheets on artificial surfaces under physiological conditions[11, 29-31]. Despite the numerous studies, imaging of S-layer structures on living cells is rarely described and remains very challenging. Dufrene et al. described the visualization of the hexagonal S-layers of *Corynebacterium glutamicum* living cells by AFM. These cells were trapped in porous polymer membranes prior to analysis[7]. This method allows for the consistent immobilization of cells sufficient enough for noninvasive in vivo imaging of the cell surface. However, in this study, only limited parts of the cell surface could be imaged. In contrast, the immobilization method developed in the present study was found to be adequate to image S-layers on the surfaces of living filamentous cells of bacilli.

AFM imaging can be used for liquid samples and requires no drying steps or contrasting treatments that may destroy or alter specimen. Drying of sample material can cause the formation of artifacts, such as cracks in the cell envelope[23, 32], flattening due to water loss from the cells, and other deformations caused by the surface tension of water during drying. Even critical point drying causes shrinkage[33]. Though not all microbes are affected[34], drying can also lead to inactivation of some microorganisms[35-37]. Only the avoidance of drying prevents such artifacts and allows for the imaging of living cells and cellular processes. However, a major challenge of AFM investigations of microorganisms in liquids is the immobilization of cells on a flat substrate. This is due to the fact that cells that are weakly adhered might be moved or even detached by the probe tip during scan. The only exceptions are cell cultures which grow epithelial the surface[38]. Numerous immobilization methods have been reported in the literature, but the application of each of these methods is organism specific. For example, Coccoid cells can be mechanically trapped in the pores of a filter membrane[39] or at lithographically patterned substrates[30]. Another approach enhances the adhesion be-

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tween microorganisms and substrate by coating the substrate with a variety of substances. Poly-L-lysine coated surfaces are commonly used for cultivating mammalian cells, but are also suitable for immobilizing these cells for AFM analyses. This kind of coating was also used for the immobilization of *Escherichia coli*, but again requires a short drying step[40]. In other studies, coating of mica surfaces with gelatin was found to be more effective for immobilizing *E. coli*, *Rhodopseudomonas palustris*, and *Staphylococcus aureus*[41]. Other methods use substrates coated with simple media (de Man, Rogosa and Sharpe broth). These also require drying and rehydration of the samples[6]. Direct chemical crosslinking methods are possible as well[42]. These provide stability for AFM experiments when preparation conditions are complex or may chemically alter the specimen. Generally, many of these methods are often adapted to specific organisms so that the immobilization of other organisms necessitates the development of new immobilization techniques or the optimization of existing ones.

Prior to this work we tried several immobilization methods with our organisms. The membrane trapping method[39] was not applicable with filamentous cells. Immobilization on gelatin[41] or poly-L-lysine[40] coated surfaces without drying was not effective. The inability to successfully and consistently immobilize organisms for AFM analysis in our laboraties has prompted the need for a new immobilization method. Attaching filamentous cells, like bacilli, to flat surfaces is difficult without the use of drying. This becomes even more challenging when trying to immobilize living, mobile cells. In the present study an adaptable method was developed that allows for the facile immobilization of a wide range of single cell organisms by means of polyelectrolyte modified surfaces combined with centrifugal sedimentation. This method completely avoids movement of the cells, thus allowing detailed imaging of unaltered microbial surfaces and extended scanning times necessary to obtain large area overview scans, such as is required for large eukaryotic single cell organisms. Polyelectrolytes bearing many uniform charged functional groups can promote cell adhesion[43, 44], and the layer-by-layer deposition of them is a well-known technique that allows for well-defined deposition of these polymers on a wide variety of substrates[45-47]. Layers can be deposited by either dip or spin coating. Dip coating is easy to perform and requires no additional equipment, and spin coating is a notably quick process. The applicability of this method was tested with Gram-negative *E. coli* BL21 for showing cell division and two

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Gram-positive bacilli recently genetically identified as *Lysinibacillus sphaericus* JG-B53[48] and *Viridibacillus arvi* JG-B58. These strains originate from a uranium mining waste pile[49] and are enveloped by a surface layer (S-layer) with enhanced heavy metal binding capacities[50]. The eukaryotic organisms *Pichia pastoris* and *Chlorella vulgaris,* which are much larger and more difficult to image via AFM were also immobilized and imaged. In this work we report a new reliable and efficient method of immobilizing a wide range of different microorganisms for the application of AFM imaging and analysis of cell surface structures on the nanometer scale.

MATERIALS AND METHODS

ORGANISMS AND CULTIVATION. Cultivation of *Escherichia coli* BL21 (DE3) was done in Luria-Bertani medium (LB) at 37°C. *Lysinibacillus sphaericus* JG-B53 and *Viridibacillus arvi* JG-B58 were cultured in 10 g/l nutrient broth medium (Mast Diagnostica GmbH; Reinfeld, Germany) at 30°C. *Pichia pastoris* was grown in Yeast Extract Peptone Dextrose medium (YPD) at 30°C. Cells of the algae *Chlorella vulgaris* Beijernick 211-11b (SAG Culture Collection, Göttingen, Germany) were grown in mineral media[51] in daylight at room temperature. Organisms were shaken in 200 ml Erlenmeyer flasks at 110 rpm.

SUBSTRATE PREPARATION. Silicon dioxide wafers were provided by AMD Saxony LLC & Co. KG (Dresden, Germany) and cut into 5 mm x 5 mm pieces. Surfaces were cleaned using the RCA-Method[52]. The polyelectrolyte coating was applied via spin coating at 6,500 rpm. Experiments were carried out with the spin coater SCI-20 (Ingenieurbüro Jörg Reinmuth, Markleeberg Germany). The multilayered coating was applied using PEI (polyethyleneimine, MW 25,000, Sigma) and PSS (polystyrenesulfonate, MW 70,000, Sigma) starting with a positively charged polyelectrolyte. The number of layers was varied from 1 to 15 layers of polyelectrolytes. A freshly cleaned wafer was placed at the spin coater after intensive rinsing with ultrapure water. A drop of 25 µl of the polyelectrolyte solution (3 g/l in ultrapure water) was placed in the middle of the spinning wafer. Dispersion of the solution took about ten seconds and was followed by the addition of 100 µl of ultrapure water. Substrates can be stored under dust free conditions at room temperature for several days.

SAMPLE PREPARATION. Prior to cell immobilization, 1.5 ml tubes were filled with 1 g molten hot glue and centrifuged immediately to form a tilted bottom. The wafer piec-

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es were placed on the solidified glue in the tubes. Microorganisms were harvested by centrifugation (8,000 x g, 4°C, 20 minutes) and washed three times with phosphate buffered saline (PBS). Optionally, the cells were fixed by adding glutaraldehyde (25% in water, electron microscopy grade, SERVA Electrophoresis GmbH, Heidelberg, Germany) to a final concentration of 5% and incubation for four hours at room temperature. Cells were washed twice with PBS after fixation. Optical density at 600 nm was adjusted to 0.05 to 0.2 with PBS. Subsequently, 250 µl of the adjusted microorganism suspension was added to the tubes. The tubes were centrifuged at 15,000 x g for at least 60 min at 4°C. After centrifugation the wafer pieces were removed from the tubes, rinsed intensively with ultrapure water to remove weakly bound bacteria, and immediately imaged with optical microscopy and AFM.

DATA EVALUATION AND BIOSTATISTICS. To verify the cell coating on the SiO_2 wafer the immobilization was performed in a 5-fold determination. The pictures for the optical microscopy were modified using a blank wafer without any adhesion of microorganisms to deduct fragments of the lens system. The camera focus was set to a wafer area of ~3.7 x 10^{-4} cm^2 area and was standardized to 1 cm^2. The statistics of the counting values were calculated with a confidence interval of 95 % using the statistical spread of the 5-fold determination and are marked as bars in the cell counting statistics.

OPTICAL MICROSCOPY. Optical microscopy was performed in 400x magnification with an Olympus BX61 Microscope (Olympus Europe Holding GmbH, Hamburg, Germany) using incident light illumination. The images were taken with the Cell^P imaging program (version 3.1, Olympus Soft Imaging Solutions LLC, Münster, Germany).

ATOMIC FORCE MICROSCOPY. AFM Imaging was done with a MFP3D Bio (Asylum Research, Santa Barbara CA). All images were taken in AC mode while the cantilever was excited by a piezo at its resonance frequency. The distance of the cantilever to the surface was determined by the oscillation damping. The cantilever of choice was the Biolever mini (Olympus BL-AC40TS-C2) with a resonance frequency of about 25 kHz in water and a stiffness of 0.09 N/m. All measurements were performed in liquid (ultrapure water, PBS or media). Therefore a closed fluid cell (BioHeater™, Asylum Research, Santa Barbara CA) was used. The cell has a volume of about 1.5 ml. The temperature of the cell content was kept constant at 30°C. Scanning speed was adjusted between 2.5 and 10 µm/s. Height images are shown

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with z-scale while z-values represent the exact topography of the surface. Amplitude (Pseudo 3D) images are shown without z-scale, which depend on scanning parameters and bear limited information.

RESULTS AND DISCUSSION.

The immobilization technique was tested with multiple organisms from different two domains of life. Two bacteria and eukaryote strains were immobilized. *E. coli*, the standard organism for genetic engineering, was tested as representative for Gram-negative bacteria. The short generation period makes the bacterium adequate for imaging cell division processes. The Gram-positive *Lysinibacillus sphaericus* JG-B53 and *Viridibacillus arvi* JG-B58 are covered by a p4-symmetry S-layer protein envelope with about 14 nm unit-cell size that is viewable only by AFM or TEM. *Pichia pastoris* is a methylotrophic yeast, which is often used as host for recombinant expression of proteins, reproduces via budding. *Pichia pastoris* has larger cells than common bacteria, but even bigger is the identically immobilized single-cell algae *Chlorella vulgaris*. Objects with increased heights are more difficult to be imaged via AFM. While AFM has its strength at the nanometer scale, organisms of a few micrometers can still be imaged, though with some limitations. The feedback loop and the Z-piezo of the AFM react every time the probe tip reaches a sample object. The taller the object is the more the time feedback loop and Z-piezo need to elevate the tip avoiding damage to the object. If scanning speed is not reduced it will result in an increased shear force applied to the object by the probe tip. These tall algae cells are therefore predestinated to test the stability of immobilization.

An appropriate density of immobilized cells is essential for proper imaging of the organisms by AFM. Low cell density prolongs the scanning time that is needed to find proper cells for further analyses, which may result in wear or contamination of the tip. Conversely, analyzing high cell density may result in cell damage due to mutual interference of the cells during the sedimentation caused by centrifugal forces. Therefore, the cell density was checked with optical microscopy prior to AFM imaging. **Figure 1** shows optical micrographs of four wafer pieces with immobilized bacteria (*Viridibacillus arvi JG-B58* A, *Lysinibacillus sphaericus* JG-53 B; *E. coli* C and D) deposited from suspensions of varying concentrations (OD_{600}=0.1 for A and C; OD_{600}=0.05 for B and D). The cells are uniformly distributed on the surface and the influence of the cell density is clearly visible. Cell suspensions with an optical density be-

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tween 0.1 and 0.05 generated the best results for the tested bacteria and were used for AFM sample preparation.

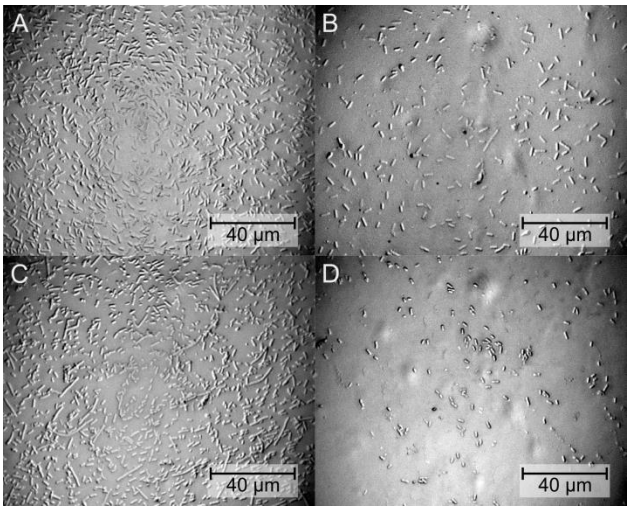

Figure 1. Microscopic images of microorganisms using incident light illumination on a silicon wafer after immobilization; (A) *Viridibacillus arvi* JG-B58, OD600=0.1 (B) *Lysinibacillus sphaericus* JG-B53, OD600=0.05 (C) *E. coli* OD600=0.1 (D) *E. coli* OD600=0.05

In addition to the experiments for the cell density, the constitution of the silicon dioxide substrate was checked to verify the ideal polyelectrolyte composition. The immobilization of microorganisms with an adjusted optical density of 0.1 were tested on silicon dioxide substrates with 1 to 15 polyelectrolyte layers and substrates without the polyelectrolyte layering. The adhesion of microorganisms on differently charged polyelectrolytes was investigated. In **Figure 2** the data evaluation of the tests with *Lysinibacillus sphaericus* JG-B53, a representative for the investigated microorganisms, is shown.

The experiments indicate that a positively charged final polyelectrolyte layer is important for the stable adhesion of bacterial cells to the substrates. This effect can be explained by the negative net charge of the bacteria cell itself. All results with the negatively charged PSS layer results in weakly bound cells in low amounts on the substrate surface. A stable cell value of approximately 2.2 x 10^7 adhered bacterial cells per square centimeter can be obtained by all odd layers of 3 to 15 as shown in **Figure 2**.

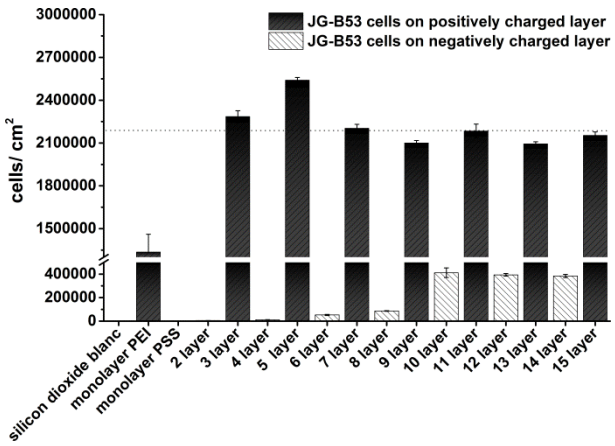

Figure 2. Total cell numbers representing effects of polyelectrolyte coatings on silicon dioxide surfaces to the bacterial cell adhesion of *Lysinibacillus sphaericus* JG-B53. Total adsorbed cells were normalized to one square centimeter depending on different constituted PE mono- and multilayer.

Although, additional microscopic analysis of marked regions on the silicon dioxide wafer have shown that the bacteria immobilized

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on substrates with lower positively charged layer numbers can be easily removed by the AFM probe tip. Therefore, we recommend for this immobilization strategy a polyelectrolyte layer thickness of 15 to generate a stable and reliable immobilization for a variety of microorganisms. Applying 15 layers enhances the surface roughness of the silicon substrate from approximately 800 pm to 3.5 nm, which is irrelevant for imaging microorganisms of a total height in the micrometer scale. Images of the silicon surfaces before and after the application of the 15 layers are shown in **Figure 3**. In **Figure 3** (C) the according surface plots are shown that clarify the surface roughness of the coated and uncoated substrates.

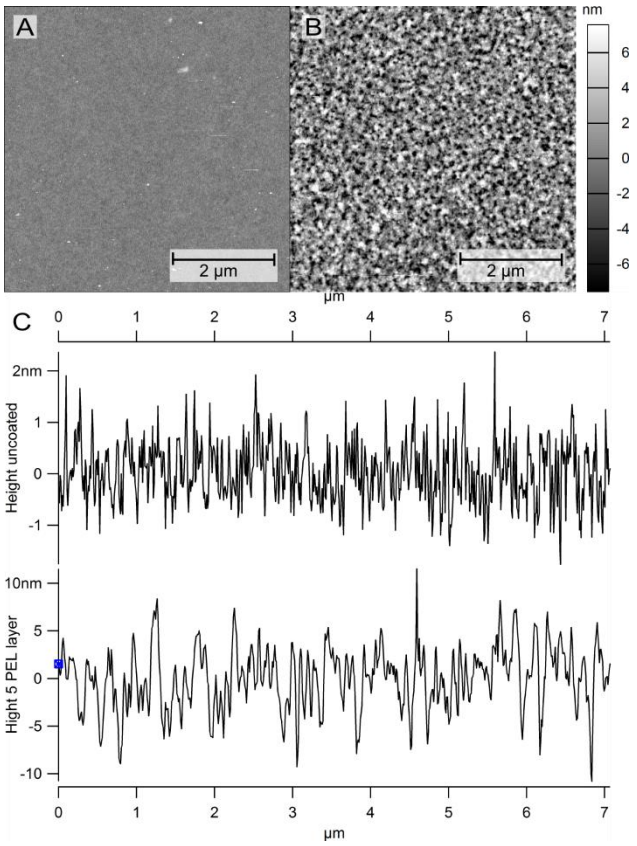

Figure 3. AFM images of silicon substrates; (A) AFM scan of plain silicon wafer, (B) AFM scan of silicon wafer after applying 15 polyelectrolyte layers, (C) Surface profile of uncoated and coated silicon wafer derived from the AFM images (A) and (B) measured from the lower left to the upper right corner.

For using AFM analyses at the nanometer scale, cells are relatively large objects. Imaging such objects with AFM can be time consuming for two reasons: scanning speed has to be reduced and the broader scanned area results in extended scan durations. A fixation of the cells with glutaraldehyde simplifies taking high quality images. Usually this procedure does not alter the details of interest, but allows for easier scanning due to an en-

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hanced stability of fixed cells. Cell movements as well as cell division are inhibited by the fixation, thus allowing a longtime scanning. Though, this prevents the observation of transient cell activities like cell division or changes in the cell surface morphology. To perform such investigations a high viability of cells and imaging in physiological conditions are required, which are not feasible when cells are fixated.

The method presented here can be applied for fixed and living cells. **Figure 4** shows AFM amplitude images of different magnifications of *E. coli* cells that were fixed with glutaraldehyde. The analyses show that even repeated scanning did not alter the sample and the cell surface remained unchanged. The immobilized microorganisms are homogeneously distributed on the surface as shown in the overview scan in **Figure 4** (A). **Figure 4**(B) shows three bacterial cells with one bacterium fixed in the state of cell division. More detailed analyses of one of these bacteria in **Figure 4** (C) presents the cell surface. AFM analyses allow the visualization of details of the cell surface on nanometer scale as presented in **Figure 4** (D). Surface features that are observed in **Figure 4** (C) are also found in the magnified area in **Figure 4** (D).

The tested microorganisms were reliably immobilized revealing long time stability in the case of glutaraldehyde fixed cells. The samples show a good stability against the scanning probe tip of the AFM, which tends to remove weakly, bound samples from the surface. Such removal of sample material was only rarely observed. In most cases bacteria were removed if stacked on top of each other where they were not properly attached to the polyelectrolyte coated surface. In contrast, no proper immobilization of *Lysinibacillus sphaericus* JG-B53 and *Viridibacillus arvi* JG-B58 using the gelatin method[41] could be observed (data not shown).

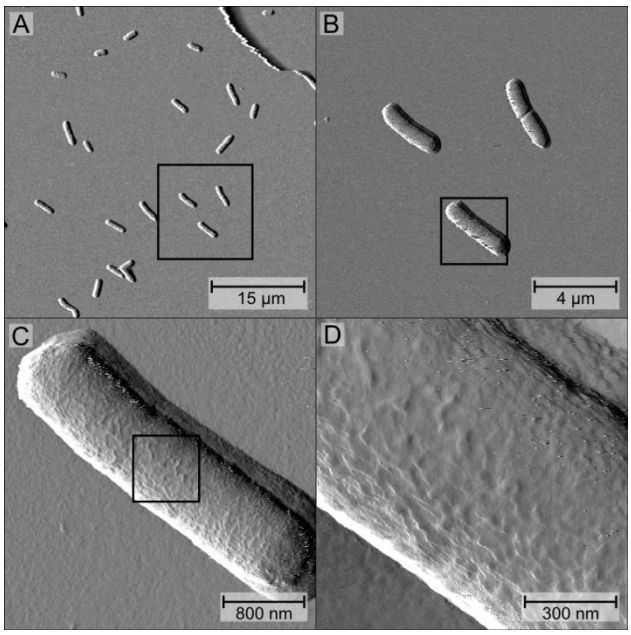

Figure 4. AFM images of *E. coli*; amplitude images, (A) 50 µm scan, (B) 15 µm scan, (C) 3 µm scan, (D) 1 µm scan.

The Gram-positive bacteria *Lysinibcillus sphaericus* JG-B53 was fixed and immobilized on polyelectrolyte coated SiO_2 substrate.

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In **Figure 5** one discrete bacterium is visible. The immobilization on a flat substrate offers the possibility of precise height measurements of the cells. The surface profile (1) of the bacteria in **Figure 5** shows a slight flattening in the middle part of the cell in comparison to the ends of the cells that are not flattened as demonstrated by the surface profile (2) in **Figure 5**.

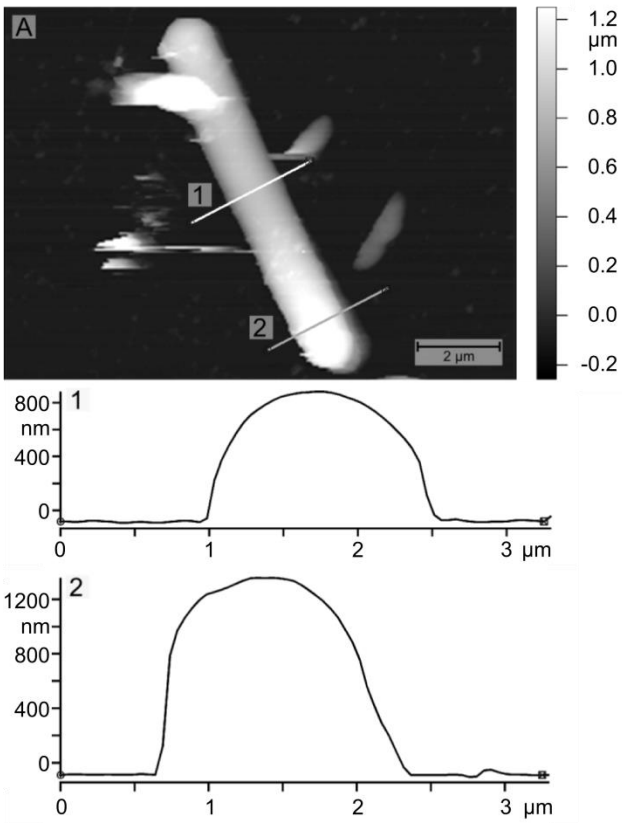

Figure 5. AFM images of *Lysinibacillus sphaericus* JG-B53; (A) height image, (1, 2) height plot along the white lines in the height image (A)

Such flattened cells were only rarely observed and there are multiple reasons for this. It can be caused by the force that is applied by the probe tip or a beginning formation of endospores. While the cells are fixed and the cantilever is very soft the influence of the cantilever is unlikely. The formation of endospores occurs only at one end of the cell and is therefore doubtful as well. The most plausible reason is the deformation of the cell by other cells during centrifugation. Strong adhered cells could be deformed by loosely adhered ones that have been removed during washing or scanning.

In further experiments eukaryotic microorganisms, represented by the yeast *Pichia pastoris* and the single cell algae *Chlorella vulgaris*, were immobilized using the described method. These eukaryotes were larger in size than the bacteria that were used, which resulted in increased shear forces and complications with the imaging. However, **Figure 6** shows that these shear forces did not cause a removal of the yeast cells, proving the good stability after immobilization. For the experiments, cells of *Pichia pastoris* were harvested in the exponential growth phase, fixed with glutaraldehyde, and subsequently immobilized. The samples included many cells with buds or recently detached daughter cells. These division processes were stopped by fixation. The sample remained stable for imaging and further investigations. The bud scars could be imaged by AFM as presented in **Figure 6** (B-D).

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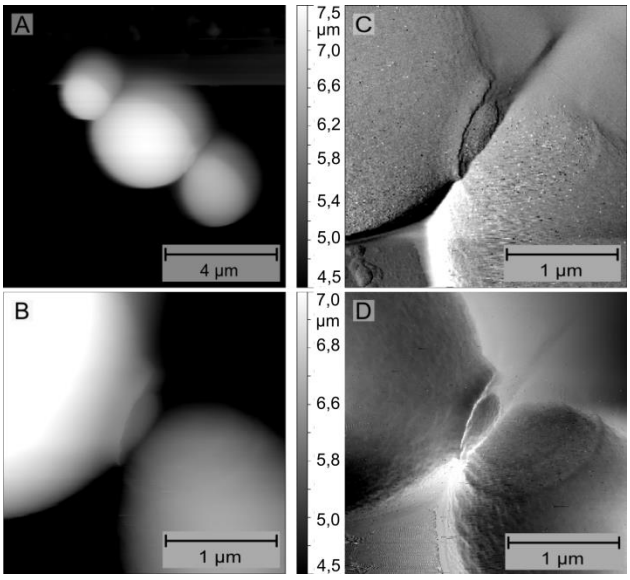

Figure 6. AFM images of *Pichia pastoris* (A) overview scan - height image (B-D) single cell scan – (B) height image (C) amplitude image (D) phase image.

Likewise, in case of the algae *Chlorella vulgaris*, the even larger single cells remained stable at the coated silicon surface. However, in some cases very thick cells of *Chlorella vulgaris* were destroyed during the scan, probably caused by the sharp probe tip. The images of intact yet fixed cells are shown in **Figure 7**.

This data reveals other difficulties of AFM analyses of biological samples. The increased height of the eukaryotic cells compared to the bacteria caused tip artifacts that are rooted within the technique of AFM and are closely related with the used cantilever type. These artifacts are visible in **Figure 7**, especially in the amplitude images in part (C). The cell is surrounded by a smooth obliqueness that does not represent the real surface topography.

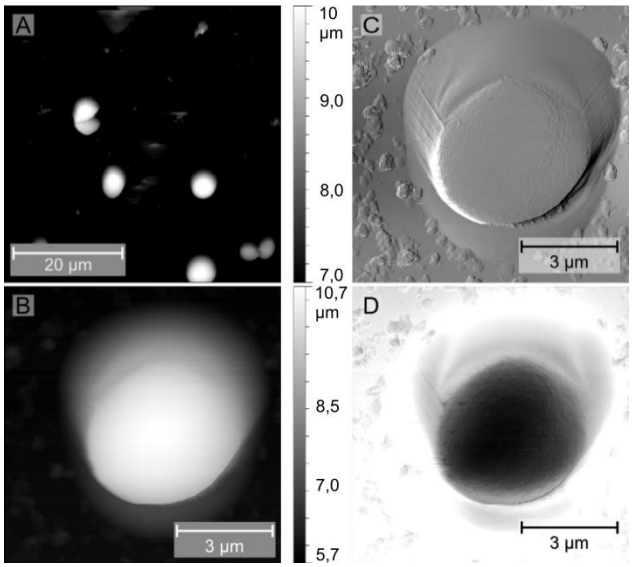

Figure 7. AFM images of *Chlorella vulgaris* (A) overview scan - height image (B-D) single cell scan – (B) height image (C) amplitude image (D) phase image.

So far described experiments included the fixation of the cells with glutaraldehyde. Monitoring of cell processes under physiological conditions by AFM analyses requires an immobilization method for living cells while keeping their viability. The developed method fulfills these requirements. Cells of *Lysinibacillus sphaericus* JG-B53 and *Viridibacillus arvi* JG-B58 were immobilized and imaged via AFM. The immobilization was persistent enough to enable the imaging of details of the cell surface on nanometer scale. **Figure 8** shows one end of a *Viridibacillus arvi* JG-B58 cell. Clearly visible is the square p4-symmetry of the S-layer lattice as well as

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areas of different lattice orientation at the convex end of the rod shaped cell.

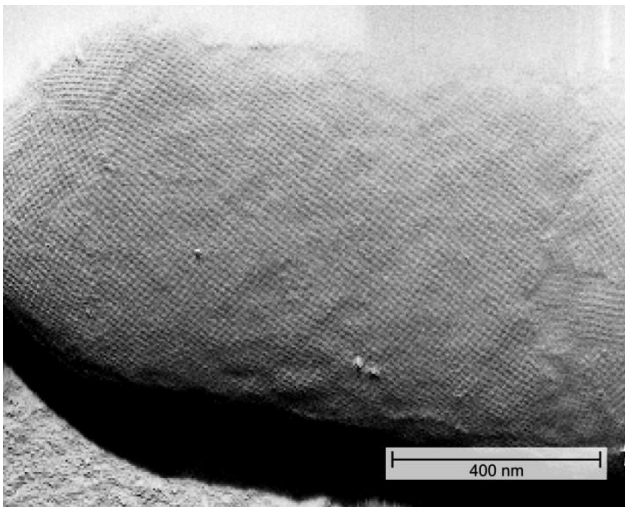

Figure 8. AFM amplitude images of *Viridibacillus arvi* JG-B58 cell with S-layer envelope.

Figure 9 shows amplitude images of a sample overview and detail scans of the cell surfaces of the two strains JG-B53 and JG-B58. The high resolution images of the detailed scans visualize the S-layer lattices on the bacterial surfaces. Although both S-layers are assembled in p4 symmetry, morphological differences, especially cavity size, are observable even on this living organism. These results substantiate the effectiveness and usability of this immobilization method.

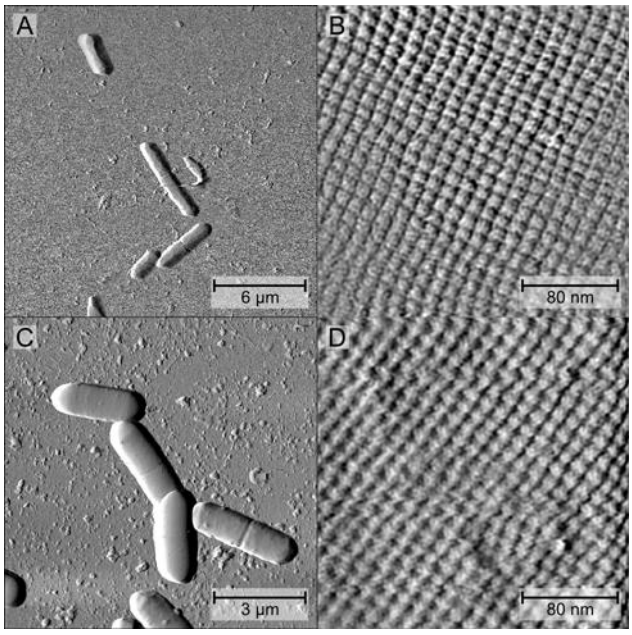

Figure 9. AFM amplitude images of cells of *Lysinibacillus sphaericus* and *Viridibacillus arvi* (A) overview scan of *Lysinibacillus sphaericus*. JG-B53 (B) detail scan of *Lysinibacillus sphaericus* JG-B53 S-layer lattice (C) overview scan of *Viridibacillus arvi* JG-B58 (D) detail scan of *Viridibacillus arvi* JG-B58 S-layer lattice (E) *Viridibacillus arvi* JG-B58 cell with visible S-layer lattice.

Another challenging task was the monitoring of cell division processes via AFM. Achieving this implies the immobilization does not affect the viability of the organisms. **Figure 10** shows three AFM amplitude images of *E. coli* that were taken with a time difference of 45 minutes. The bacteria that were harvested in exponential growth phase were immobilized and imaged in LB-media at 30°C. In this sequence multiple cells are dividing, which is marked with arrows. These results prove the method to be an ideal for

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AFM imaging of living and fixed microorganisms.

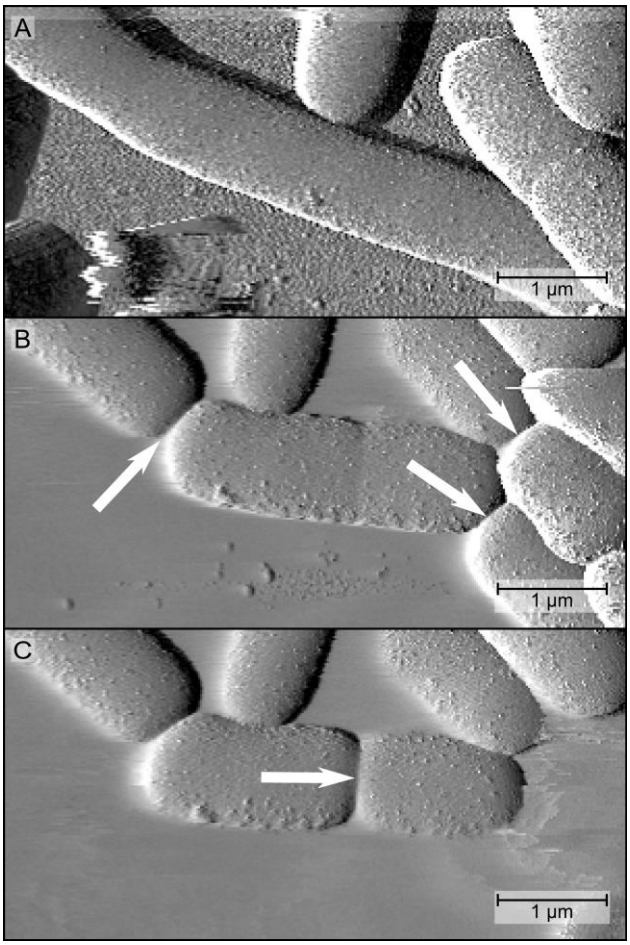

Figure 10. AFM amplitude images of living *E. coli* (amplitude image, time difference between images 45 min) immobilized at polyelectrolyte coated silicon wafers. Viability is proved by cell division.

Some general considerations relating to the immobilization method and AFM analyses have to be taken into account. Firstly, a proper cell density is substantial for precise imaging without artifacts. A confluent film of microbes as shown in **Figure 11** (A) simplifies localization of desired objects, whereas the usage of a suspension with too high cell density may result in the stacking of cells leading to cell deformation as shown in **Figure 11** (B). Cells that are not properly attached to the coated surface are removed and expose the subjacent deformed cells. This may result in cell destruction or unwanted behavior of cells.

Secondly, general issues connected with AFM imaging have also to be considered. AFM utilizes very sharp tips for surface recognition. These tips are pyramidal and may have edges that can introduce artifacts to the scan as shown in **Figure 11** (D). The sharpened tip of the Biolever mini is 3 µm in height according to the datasheet. The tip is followed by a pedestal shown in the SEM image in the official datasheet of Olympus BL-AC40TS-C2 cantilever. If the tip is slightly damaged after scanning, the available tip height will be reduced resulting in a reduced aspect ratio. **Figure 11** (E) presents some resulting artifacts, in this case double images that are also visible in **Figure 7**. These considerations were confirmed by a 3D reconstruction of the tip in **Figure 11** (C). Such problems mostly occur when whole cells are scanned or overview scans are performed. Detail scans of the cell surface are not or less affected by these tip problems.

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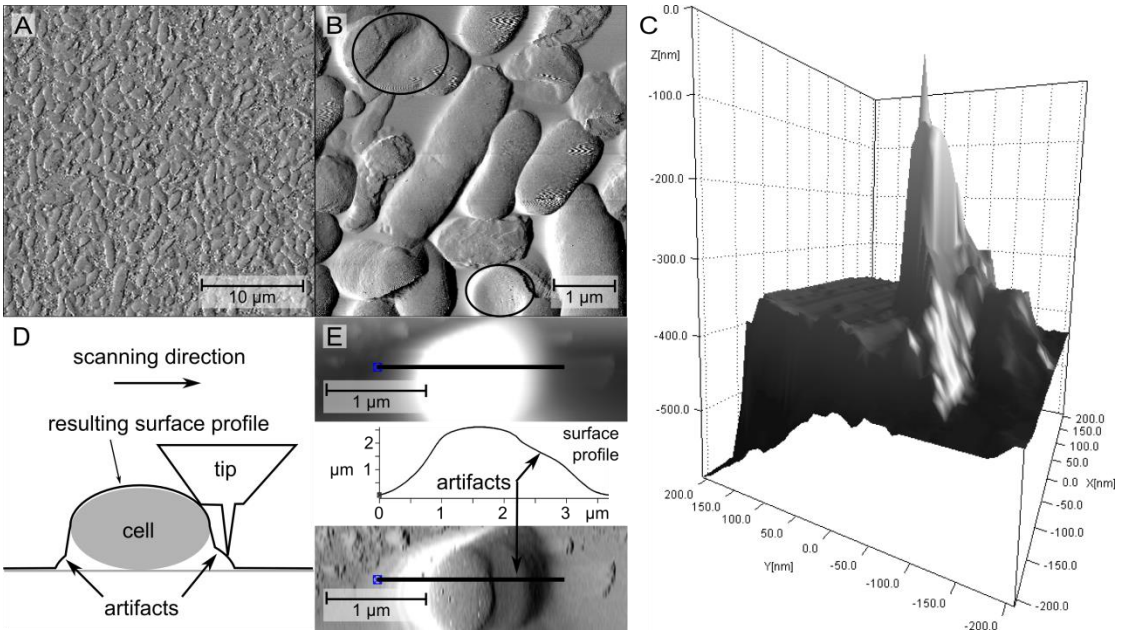

Figure 11. Origin of artifacts. (A, B) AFM image of *E. coli* cells sedimented from a too thick suspension resulting in very densely packed cells on the surface (A) overview (B) detail scan revealing damaged cells (black circles). (C) Tip reconstruction (SPIP, Image Metrology) from a worn tip using the *C. vulgaris* image (**Figure 7**). (D) Scheme of origin of tip artifacts. The tip shape influences the scanned profile; (E) AFM height image, height plot and amplitude image of a *Chlorella vulgaris* cell showing scanning artifacts illustrated in (A).

Thirdly, the stability of the analyzed cells plays a major role in obtaining AFM images of high quality. Cell stability depends on a several factors. Most importantly is the type of organism being investigated by AFM. Another stability influencing parameter is the surrounding medium, especially its ionic strength, pH-value, and osmotic potential. The softness of cell surface is organism-dependent and affects the success of scanning. Stability of soft cells can be increased by fixation of the cells with agents such as glutaraldehyde. While imaging is enhanced, fixation does not influence immobilization efficiency.

CONCLUSION

All tested organisms could be reproducibly immobilized and were stable over several hours of scanning. Polyelectrolytes provide an excellent adhesion between the substrate surface and the sample by their multiple charges. The polyelectrolyte coating can also be applied by simple dip-coating[47]. But requires more time for 15 layers. Further, all tested cells, including the large cells of algae, did not move on the silicon substrates in spite of shearing forces of the probe tip and repeated scans. The long-term stability of the sam-

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ples was verified by performing wide area scans with scanning durations of several hours. In addition, fixation with glutaraldehyde did not affect the surface structures of the microorganisms as documented by the S-layer images derived from different fixed bacilli. The presented work describes a facile yet efficient method that allows the immobilization of living organisms, thus making them available for further AFM analyses. These samples are stable enough to perform scans of microbial surfaces with very high resolution as demonstrated by the S-layer images of bacilli. Furthermore, the technique offers the possibility to monitor microbial activity by the high resolution of AFM as shown by the imaging of the division of *E. coli* cells. The immobilization of cells on flat substrates that prevents cells from drying-out allows accurate height measurements as well as good estimations of cell shapes. This also allows for the imaging of whole cells in contrast to the membrane trapping method[39]. Not only can this method be utilized to investigate microorganism from the micro to the nanoscale, but it also can be used to examine microorganisms in samples that naturally contain low amounts of cells, such as water samples, without the need of previous cultivation, whichmay change the microbial diversity of the sample. Thus, the method allows for the imaging of slowly growing microbes or organisms that are difficult or impossible to cultivate.

AUTHOR INFORMATION

Corresponding Author

Tobias Günther
Helmholtz-Zentrum Dresden-Rossendorf,

Helmholtz Institute Freiberg for Resource Technology, Bautzner Landstraße 400, 01328 Dresden, Germany

eMail: t.guenther@hzdr.de
Phone: +49 351 260 2051
Fax: +49 351 260 *12951*

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