Immobilizing live bacteria for AFM imaging of cellular processes

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A B S T R A C T
Coccoid cells of the bacterial species Staphylococcus aureus have been mechanically trapped in lithographically patterned substrates and imaged under growth media using atomic force microscopy (AFM) in order to follow cellular processes. The cells are not perturbed as there is no chemical linkage to the surface. Confinement effects are minimized compared to trapping the cells in porous membranes or soft gels. S. aureus cells have been imaged undergoing cell division whilst trapped in the patterned substrates. Entrapment in lithographically patterned substrates provides a novel way for anchoring bacterial cells so that the AFM tip will not push the cells off during imaging, whilst allowing the bacteria to continue with cellular processes.

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1. Introduction

Atomic force microscopy (AFM) is a scanning probe technique widely used in research and industrial applications for visualizing surfaces at the nanoscale as well as measuring surface physical properties. Even though electron microscopic techniques like transmission electron microscopy (TEM) or scanning electron microscopy (SEM) can be utilized for similar surface investigations, the high vacuum environment and specific sample preparation required, prevents the real time imaging of dynamic systems and following their processes in situ. AFM, with its ability to obtain high resolution images under ambient air or fluid conditions, has, therefore, been used to follow kinetic processes in many systems ranging from polymeric and crystalline materials [1–3] to cellular membranes and biomolecules [4–7].

Following dynamic cellular processes in microbiological systems like bacterial cells in their native, aqueous environment is important for obtaining more information regarding the architectural dynamics, surface molecular interactions, etc. of the microbes. The medical and clinical relevance of such studies have spurred intense research, with a considerable focus in recent times on using the high resolution capability of AFM to probe the microbial world [8]. However, AFM imaging of bacterial cells in liquid in order to visualize live cells performing natural cellular processes has proved difficult. Even whilst imaging in air, due to the turgid shape of the live bacterial cell, the contact area between the cell and the substrate is minimized, thus making it easy for the AFM probe to detach the cell from flat substrates whilst scanning. When imaging under liquid, the mobility of live cells causes an additional hurdle. Immobilizing the cells is therefore one of the primary challenges encountered while imaging bacteria.

Bickerstaff [9] lists adsorption, entrapment, covalent binding, cross-linking and encapsulation as the five principal methods of immobilizing microbial cells. As AFM is a surface imaging technique, encapsulation would not be an ideal method for studying cells. The reactive groups used in covalent binding and the reagents used for cross-linking are known to affect cell viability, making these two methods useful for immobilizing non-viable cell preparations [10]. For immobilizing viable cells, entrapment and adsorption techniques are found to be more effective. Mechanical entrapment in porous membrane filters [11–16] or aluminium oxide filters [17], soft gels like agar [18], agarose [19], gelatin-coated mica surfaces [20,21], surfaces treated with poly-γ-lysine [22–24], polyethyleneimine [25], silane trithoxysilyl-propyl-diethylenetriamine [26], etc. have been used for immobilizing cells in order to carry out AFM studies under...
physiological conditions. Pore size compatibility, depth of pores and confinement effects are major issues to be addressed while trapping the cells in membrane filters. It is difficult to follow active processes like cell division while a sizable portion of the cell is trapped inside the pore. For cells trapped in soft gels to be imaged with AFM, the gel layer needs to be very thin so that the cells will not be pushed deeper during scanning, especially with contact mode. Entrapment in soft gels might also alter the bacterial surfaces which could lead to artefacts in AFM images.

In this paper, we present a novel method of immobilizing bacteria on inert surfaces for in vivo AFM imaging of coccoid bacterial cells. The microbial system under study is Staphylococcus aureus, some strains of which are virulent human pathogens causing a range of illnesses from minor skin infections to life threatening diseases. This bacterial species has been the subject of intense research in recent times as its antibiotic resistant strain methicillin-resistant Staphylococcus aureus (MRSA) is one of the main causes of nosocomial infections worldwide [27–30]. Lithographically patterned surfaces with hole arrays are used to trap the cells and at the same time allow the cells to continue with their cellular processes. Trapping the cells in the holes also has the additional advantage of reducing the vertical height to be traversed by the AFM probe in order to scan over ~1 μm diameter cells. Using this technique of immobilization of cells, we were able to follow clearly the process of cell division in S. aureus under AFM.

2. Materials and methods

2.1. Bacterial samples

S. aureus (SH1000) cells were grown in brain heart infusion (BHI; Oxoid) liquid culture at 37°C until the optical density of the liquid medium at 600 nm (OD_{600}) had reached 0.8. The cells were then harvested by centrifugation at 5000 rpm for 10 min at 4°C and washed twice using double distilled water (ddH2O). A sample of cells was then resuspended in ddH2O to an OD_{600} of 0.8.

2.2. Substrate preparation

The techniques of contact mask photolithography and laser interference lithography were used in order to make arrays of holes patterned on a positive photoresist material. The contact mask photolithography procedure is as follows: Commercially obtained silicon wafers are subjected to oxide deposition in a plasma-therm 790-chamber for 25 min in order to attain a micron thick SiO_{2} layer on top. The wafers are spin-coated first with an adhesion promoter (hexamethyldisilazane) and then with a positive photoresist (BPRS100) at 4000 rpm for 30 s. After prebaking and edge bead removal, the photoresist is exposed to UV light from a Karl Suss MJB3 UV400 Mask Aligner through a custom built photomask for 0.4 s. The sample is then rotated by 90° and the exposure is repeated. The development of the photoresist resulted in the creation of a square lattice comprising of holes of ~300 nm depth and diameter of ~1.2 μm.

Most of the substrates used in the study have been used without etching, unless stated otherwise. CML and LIL denote substrates made using contact mask photolithography and laser interference lithography, respectively.

Fig. 1. An AFM height image showing the CML substrate imaged in air. The scale bar represents 10 μm.

Fig. 2. An AFM deflection image showing S. aureus cells on an LIL grid imaged in air. The scale bar represents 1 μm.

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2.3. AFM measurement

A drop of the bacterial suspension was deposited on the lithographic grid and allowed to dry for 15–20 min. This drying step is not a strong desiccative process that completely dehydrates the sample surface but rather a mild procedure which removes a large volume of the liquid droplet by slow evaporation, but leaves the sample surface damp and allow cells to settle gradually into the surface filled with holes. The substrate was washed with water to remove the excess material and placed on a home-built liquid cell chamber. The set-up was placed on the sample stage of a Dimension 3100 AFM with Nanoscope IV controller (Veeco Instruments). The cells trapped in the hole arrays patterned on the photoresist were imaged in air. BHI broth was introduced to flood the chamber and immerse the substrate in order to provide the growth medium for the cells. Measurements in air (before the introduction of the BHI broth) and under liquid were carried out in contact mode using the triangular silicon nitride cantilevers (with a nominal spring constant of 0.01 N/m from Veeco) mounted in a fluid cell. Topographic and deflection data were collected simultaneously.

3. Results and discussion

An AFM height image of the bare CML substrate imaged in air is given in Fig. 1. When the lithographic substrates containing the *S. aureus* cells are imaged in air, groups of connected cells can be

![Fig. 3](imageurl). (a) An AFM topography image showing *S. aureus* cells trapped in holes of a CML substrate imaged under the BHI solution and (b) a section analysis taken along the white line drawn across the image. Dark to bright colour variation in the AFM image represents a change in height of 2.5 μm and the scale bar represents 5 μm.

![Fig. 4](imageurl). AFM images of an *S. aureus* cell undergoing division. Image (a) is taken in the height mode and the scale bar represents 3 μm. 4(b)–(l) are deflection images of the cell [arrowed in image (a)] and the scale bar represents 1 μm.

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seen with many of them outside the holes. Fig. 2 shows an AFM deflection image of S. aureus cells on a LIL grid imaged in air. After the introduction of the BHI solution into the liquid cell chamber, most of the bacterial cells were displaced and floated off, except those that were held firmly in the holes. Fig. 3 shows an AFM height image of those cells trapped in the holes of a CML substrate imaged in liquid and a section analysis taken along the white line drawn across the height image. It is clear from the AFM image that most of the holes in the substrate are filled with cells and the section analysis shows that the depth of the holes is almost equal to half the height of the bacterial cell.

Several factors will influence the trapping efficiency of the holes. The larger the total area of contact of the hole with the bacteria, the greater is the total adhesive force, and hence, more effective the trapping. However, creating a perfect fit is neither possible (the cells have slightly varying sizes) nor desirable (a perfect fit leaves no room for growth). A tapered hole, such as those star-shaped (Fig. 4a), allows multiple surface contacts between a bacterial cell and the hole walls regardless of its precise size, while still giving room for growth. The depth of the hole will control the magnitude of the force component tending to lift the bacterium out of the hole. The deeper the hole, the smaller will be this component. However, if the holes are too deep, the low aspect ratio of the tip and the large discontinuity in height will cause problems for imaging and it will no longer be possible to image the entire top surface of the bacterial cell. We have found that a good compromise is a hole depth that is comparable to the bacterial half height.

Once the cells were trapped in the holes under the BHI solution, we were able to follow the process of cell division. Fig. 4 shows a series of AFM images of a cell trapped in a star-shaped hole undergoing division and imaged in liquid at room temperature (~25 °C). It took more than 2 h to follow the cell changes from Figs. 4(b) to (h) as the temperature was not the optimal one (37 °C) for cell growth. However, it is immediately clear from the images that the cells remain viable and are able to grow and divide whilst being imaged. By the time the image capture of Fig. 4(h) was complete, the dividing cells had grown so much out of the hole that the AFM tip finally pushed one of the protruding cells (the one on the bottom in the images) out of the hole as can be seen in the subsequent scan [Fig. 4(i)]. The other cell (the one on the top in the images) was pushed deeper into the hole as there was now ample space inside the hole to accommodate it [Figs. 4(j)–(l)].

The fracture of the septum (the partition separating the two daughter cells during cell division) took place between the image capture of Figs. 4(d) and (e) and at a rate faster than that could be followed by conventional AFM. Figs. 5(a)–(d) are magnified versions of Figs. 4(b)–(e). It can be seen from the images 5(a) and (b) that tiny crevices appeared at the septum which grew deeper into the cell. Touhami et al. [12] have also reported similar fracture features at the septum while studying S. aureus cells trapped in a porous membrane, while Francius et al. [31] have revealed the septum by degradation of the cell wall with lysostaphin. The crevice formation is probably due to peptidoglycan (the structural component of bacterial cell wall that made up of glycan strands that are cross-linked by peptide side chains) hydrolytic activity at the septum. Previously, the autolysin ATL has been shown to accumulate at the septum and to be involved in cell separation [32,33]. The peptidoglycan in between the cracks seems to stretch along the cell wall perpendicular to the septal ring as the division progresses and there is some indication of plastic deformation prior to the fracture. It appears that the cell wall at the septum is being torn apart rather than being simply degraded away. From Fig. 5(d), it is clear that the two daughter cells retain on their cell wall the scars left during the septal fracture.

Interestingly, as the cells grow on the patterned substrates, the shape of the holes in which they have become lodged determines some morphological parameters of the cell. This can be seen by an apparent square shape of the S. aureus cell as it grows out of the star-shaped hole [Figs. 4(g) and 5(d)]. Such an effect of spatial constriction on cell morphology has been previously noted for Escherichia coli [34]. This is likely to occur by peptidoglycan cross-links being inserted at the time of synthesis which effect later mature cell morphology.

The cell growth occurs not only in the x–y plane, but also in the z-direction. The growth of the dividing cell in the z-direction is followed in the graph (Fig. 6), giving line profiles taken at the midsection of the images shown in Figs. 4(b)–(h). The legend in chart 6 gives the time in seconds after the image capture of Fig. 4(a). From the graph, it is evident that the cell almost doubled in height after 2 h.

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S. aureus cells are known to divide in successive perpendicular planes [35]. Fig. 7 shows a series of AFM deflection images showing the division in alternating perpendicular planes. The liquid cell set-up was placed on a Linkam hot stage and heated to a temperature of 37°C. There was a time gap of 16 min between the capture of images Figs. 7(a) and (b). The rest of the images [Figs. 7(c)–(e)] were captured at 4 min intervals. The white arrow in the images points to the septal ring formed when the cell had started to divide prior to entrapment. Once the dividing cell was trapped in the hole, we were able to capture the subsequent division by one of the daughter cells in a plane perpendicular (indicated by the black arrow) to the first septal ring. The images show a slight drift, which might be due to the mechanical instability associated with the coarse positioning of the liquid cell, along with the connecting tubes for liquid transfer, on the Linkam silver block. A schematic diagram illustrating the successive cell divisions is shown in Fig. 7(f).

A mild drying step as mentioned in the Materials and Methods section was used to allow the cells to settle into the holes. Since the cells were found to engage in an active process like cell division while trapped in the lithographic grids, one can conclude that the viability of these cells was not compromised by this method of immobilization. We have only used gram-positive bacteria which are more resistant to drying than gram-negative bacteria. It can be seen that the cells were not cramped inside the holes; the size and the shape of the holes gave the cells freedom to carry on with their natural behaviour. The inert substrates also made sure that the cell surfaces were not altered chemically by contact during entrapment.

These substrates can be used in AFM studies for monitoring the activity of the whole cell under different conditions, and the response of the cell on changing its environment such as the introduction of antibiotics. It is also possible to change the surface chemistry of the substrates to see the response of the cells while trapped in holes with different surface properties. The principal advantage of this approach compared to other methods of bacterial immobilization, such as trapping in pores, is the control over the size, shape and depth of the holes that it provides. These factors can straightforwardly be tuned to allow different sizes of bacteria, or even bacterial colonies, to be immobilized, and, through tuning the depth of the hole, maximizing the area of the bacteria that can be imaged. Different patterns on the silicon substrate can be fabricated for cells of different shapes. For example, rod-shaped cells like Bacillus subtilis, which form short chains on division can be trapped in long grooves instead of holes and imaged. We are currently fabricating grooves on CML substrate for the entrapment of B. subtilis.

4. Conclusions

We have developed patterned surfaces using lithography in order to mechanically trap live bacterial cells, thereby immobilizing them for AFM imaging under growth medium, but at the same time not interfering with their cellular physiology. This non-invasive method of trapping the cells has allowed us to follow closely a dynamic cellular process i.e. cell division. We have seen that the septal fracture during division is an intricate process involving peptidoglycan stretching across the septum. Detailed examination of these live microbes under AFM is sure to provide new insights into the life cycle of these single-cell organisms.

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References


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