Nanomechanical Measurements of Viscoelastic Properties of Bacterial Cells: Effect of Antimicrobial Peptides and Challenges Associated with Measurements in Buffer

by

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Abstract

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The effectiveness of antimicrobial compounds can be easily screened; however, their mechanism of action is much more difficult to determine. Many compounds act by compromising the mechanical integrity of the bacterial cell, and we have developed an atomic force microscopy (AFM)-based creep-deformation technique to evaluate changes in the time-dependent mechanical properties of bacterial cells upon exposure to antimicrobials.

Measurements revealed large changes to the viscoelastic parameters including a distinctive signature for the loss of integrity of the bacterial cell envelope. This technique provides unique insight into the kinetics and action of antimicrobials on bacteria. Initial experiments were performed in Milli-Q water on Pseudomonas aeruginosa PAO1 bacteria, which can withstand large osmotic pressures. Subsequent experiments addressed the challenges of performing AFM creep deformation experiments on live cells in PBS buffer. We describe measurements of, and improvements to, the stability of the experimental set-up in an effort to facilitate these challenging experiments.
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Chapter 1

1 Introduction

1.1 Bacterial Cells

Bacteria are microorganisms that have evolved over 3.5 billion years and are responsible for a wide range of phenomena in the world around us, from causing diseases to helping digest food to shaping the surface and sub-surface of the earth. Bacterial cells come in many shapes, from rod-shaped to spherical to spiral to doughnut-shaped to crescent-shaped. There are even square-shaped bacteria that exist in extreme environments [1]. Despite their different shapes, they share a common feature: they are all small. Typically, a cell has dimensions that are approximately 1 µm, occupying a volume of about 2.5 µm$^3$ and weighing only 1 pg = 10$^{-12}$ g (wet weight).

There are two main classifications of bacteria according to how they react to the Gram stain [2]. Gram-negative bacteria have a multilayered cell envelope made up of an inner phospholipid membrane, an outer asymmetric membrane that contains a significant number of lipopolysaccharides, and a thin intervening periplasmic space that contains the peptidoglycan sacculus [3]. The peptidoglycan sacculus is a cross-linked polymer mesh that stabilizes the cell wall against the internal turgor pressure of the cell. Gram-positive bacteria lack an outer membrane and have a much thicker layer of peptidoglycan (20-80 nm) that allows them to withstand larger turgor pressures [3].

*Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1 are Gram-negative, rod shaped bacteria which have a diameter of approximately 0.5 µm and a length of approximately
2.5 µm. *E. coli* K12 is a non-pathogenic lab strain that serves as a model organism for Gram-negative bacteria [4]. *P. aeruginosa* PAO1 is an opportunistic pathogenic strain that primarily infects patients who are immunocompromised, particularly those with cystic fibrosis [5].

*P. aeruginosa* exhibit twitching, a form of surface motility that is mediated by helical adhesive protein polymers several microns in length called Type IV pili (T4P) which act as grappling hooks for the cell. T4P are extended and retracted by complex motor protein assemblies embedded in the cell wall. The PilT protein subunit of the T4P motor protein complex has been shown to regulate retraction of T4P. As such, a *P. aeruginosa* bacterial cell with a PilT knockout cannot retract its pili and exhibits longer pili present in greater numbers compared to the wild type [6].

### 1.2 Antimicrobial Resistance

Great advances have been achieved in controlling bacterial infections through the use of both natural and synthetic antimicrobials. This is one of the major accomplishments of modern medicine. However, many bacteria have adapted to the overuse of these antimicrobials, developing resistance that renders the compounds ineffective [7,8]. This adaptability of bacteria poses a serious threat to humans as antimicrobial-resistant “superbugs” emerge. A 2013 report by the Center for Disease Control stated that each year in the United State alone, there are over 2 million patients infected with antimicrobial resistant bacteria resulting in 23,000 deaths [9]. Because of this, there is a pressing need to develop novel antimicrobials that are not susceptible to the development of resistance, and to obtain an in-depth understanding of their mechanism of action.
1.3 Antimicrobial Compounds

Nature has developed a broad range of compounds that disable bacteria (bacteriostatic compounds) and kill bacteria (bactericidal compounds). Many of these compounds are extracted or derived from bacteria that produce the compounds to outcompete other types of bacteria. Antimicrobial compounds typically target a structural element of bacterial cells, e.g. a component of the bacterial cell envelope, or a process within the cell, e.g. inhibiting DNA, RNA, cell wall or protein synthesis [10].

Cationic antimicrobial peptides (CAPs) form a promising class of antimicrobial compounds. Although molecular-level descriptions of the mechanism of action of CAPs on bacterial cells are lacking, it is generally recognized that the cationic peptides bind electrostatically to the anionic cell surface [11,12]. Many CAPs achieve their antimicrobial action by permeabilizing the cell membranes, however other CAPs translocate across the cytoplasmic membrane where they disrupt intracellular processes [12]. The permeabilization of cell membranes by CAPs has been described in a number of models [12-14]. Whereas the efficacy of antimicrobial action of CAPs has been studied extensively on living cells, most experimental studies of the mechanism of action, e.g. the binding and/or partitioning of CAPs, have been performed on model membranes because of the intrinsic complexity of interpreting the measurements [14-20].

1.2.1 Polymyxin B (PMB) and Polymyxin B Nonapeptide (PMBN)

PMB and PMBN are two closely related cationic peptides that have very different efficacies in killing bacteria. The chemical structure of PMB is a cyclic ring of seven amino acids (dia 1.25 nm) that are connected via two other amino acids to a fatty acyl tail 7 carbons in length (Figure 1). PMBN has an identical structure to PMB, however the fatty acyl tail has been
enzymatically cleaved (Figure 1) [21]. The large number of positive charges in the ring structure of both polymyxins (+5 for PMB and +4 for PMBN) allows the antimicrobial to penetrate the lipopolysaccharide layer and interact with the negatively charged lipid A head group. These compounds act by removing lipopolysaccharides from the outer membrane, creating small holes. It is thought that PMB then inserts its fatty acyl tail into the inner membrane to form pores, allowing PMB to enter the cytoplasm. Once the inner membrane has been permeabilized, molecules from within the cell can leak out, causing cell death [7]. Clinical use of PMB is limited because it has high nephrotoxicity [22]. The minimum inhibitory concentration (MIC) of PMB for both *P. aeruginosa* PAO1 and *E. coli* K12 is ~2 µg/mL [23]. Because PMBN lacks a fatty acyl tail, it cannot insert into the inner phospholipid membrane and thus it acts solely on the outer membrane. As a result, it has low bactericidal activity. PMBN concentrations of 50. µg/mL compromise the outer membrane of *P. aeruginosa* PAO1 and *E. coli* K12 and sensitize the Gram-negative bacteria to antimicrobials that target the peptidoglycan sacculus [24]. Antimicrobials that are typically used against Gram-positive bacteria include penicillin and vancomycin. These antimicrobials function by disrupting the maintenance of the peptidoglycan layer by binding to and inhibiting the protein responsible for covalently connecting peptidoglycan polymers [25].
Figure 1.1: Chemical structure of antimicrobial compounds. Structures of polymyxin B (left) and polymyxin B nonapeptide (right). The locations of positive charges (amine groups) are indicated. Figure adapted from [24].

1.3 Atomic Force Microscopy

1.3.1 Basics of Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a powerful experimental tool that can be used to image materials and measure forces on the nanoscale. AFM has the distinct advantage over other high resolution imaging techniques in that it is capable of imaging materials in aqueous conditions, allowing in situ studies of biological molecules, aggregates of biological molecules, and live eukaryotic and prokaryotic cells.

In an AFM experiment, a flexible cantilever with a length of approximately 100 μm and a chemically etched tip (typically pyramidal or spherical in shape) near the free end of the cantilever is translated toward the surface of interest. The cantilever acts as a spring and bends in
response to forces between the tip and the underlying surface, such as electrostatic, van der Waals, and hydration forces in liquid. The bending of the cantilever is measured by tracking the deflection of a laser beam reflected from the back of the free end of the cantilever, using a four-quadrant photodetector. Changes in the vertical and lateral deflections of the cantilever can be inferred from changes in the intensities of light detected in the four quadrants of the photodetector. These changes in deflection can then be related to changes in the forces applied by the cantilever on the surface. Although interaction forces between the tip and the sample are often very small (several nN), the tip may be chemically functionalized to enhance specific interactions (up to µN). As the tip approaches the surface and its displacement into the surface is resisted, a rapid increase in cantilever deflection is observed, indicating that the tip is engaged to the surface.
Figure 1.1.2: Schematic diagram of the deflection of an AFM cantilever that is measured as a change in the voltage of a photodiode caused by a displacement of a laser reflected onto the photodiode from the back surface of the end of the AFM cantilever.

AFM cantilevers are typically fabricated on a small chip that is then mounted onto the AFM stage in which translation is achieved by controlling the voltage applied to a piezoelectric crystal. Millivolt changes in potential across the z-piezoelectric crystal correspond to nanometer changes in the vertical displacement of the tip. X- and y-piezoelectric crystals are used to translate the tip across the surface. A feedback loop that has the photodetector signal as input and the z-piezo voltage as output is used to hold either the deflection or vertical displacement of the cantilever constant while measuring the topography of the surface with nanoscale precision.

Since the cantilever can bend in response to forces applied to the end of the cantilever, it is useful to measure its spring constant to directly relate the cantilever deflection to a force between the surface and the tip. This is done by measuring a force indentation curve on a bare
glass slide. This measurement of the tip deflection can be related to the change in voltage across the photosensitive detector by a constant that has units of nm/V. The noise power spectrum of the oscillating cantilever can then be fit to extract the voltage (units of V/nN) measured across the photodetector that corresponds to the force applied to the tip because of the Brownian motion of the fluid in which the tip is suspended. These two factors can be combined as a measure of the stiffness of the cantilever, which is typically 100 pN/nm [26].

1.3.2 Contact Mode AFM

The simplest AFM imaging mode is contact mode, in which the tip is translated directly into contact with the underlying surface. Tip engagement with the surface is achieved by extending the z-piezo until the displacement of the tip is halted by the sample, and the cantilever has experienced a prescribed deflection. As the x- and y-piezos raster the tip across the surface, changes in the height of the sample result in changes of the vertical deflection of the cantilever as measured by the photodetector. The length of the z-piezo is then adjusted to maintain a constant vertical deflection, and changes in the displacement of the z-piezo are recorded as an image of the surface topology of the sample.

1.3.3 Intermittent Contact AFM

AFM imaging in contact mode can apply forces to the surface that are large enough to damage the surface, or dislodge objects from the surface. To reduce the amount of contact between the AFM tip and the surface, intermittent contact AFM can be used. In intermittent contact mode, an alternating voltage is applied to the z-piezo, inducing an oscillation in the cantilever. The amplitude, frequency and phase of the oscillation of the cantilever can be determined by measuring the time varying deflection of the laser light on the photodetector. The amplitude of the oscillation decreases as the tip is brought closer to the sample. An image is
collected by measuring the change in displacement of the $z$-piezo as the amplitude is held constant, and the $x$- and $y$-piezos raster the tip across the surface of the sample. Since the tip is only in contact with the surface of the sample for a small part of the oscillation, and the oscillation of the tip is rapid compared to the $x$- and $y$-displacements, only small vertical and lateral forces are applied to the surface. The set point amplitude may also be optimized to minimize the force applied to the sample by starting with a set point near the target amplitude, resulting in a noisy image, and then lowering the set point by small amounts until significant features of the sample are visible.

1.3.4 AFM Force Spectroscopy

Force spectroscopy measurements are performed by pressing the AFM tip onto a surface, and then retracting the tip from the surface. This type of AFM experiment has been used extensively to measure the elastic stiffness of sample surfaces [27], and to mechanically unfold protein molecules [28]. The magnitude of the set point force, the indentation of the sample and the dwell time between the tip and the surface can be controlled. Below we describe an AFM creep deformation experiment that allows the determination of the time-dependent or viscoelastic properties of bacterial cells.

1.3.5 AFM Force Mapping

Several AFM manufacturers have developed an automated method to collect force indentation curves at each point of a high-resolution, two-dimensional $x$-$y$ grid (force mapping). One such example is the Quantitative Imaging (QI) mode implemented in JPK AFMs. At each point on the grid, a proper force curve is collected, and the tip is fully retracted from the surface before it moves to the next grid point, minimizing the lateral force applied to the sample. This allows the imaging of objects that are only weakly adhered to the underlying surface, such as
bacterial cells in buffer. Each force indentation curve can be analyzed to obtain the height, stiffness and adhesion of the sample at each point in the image.

**Figure 1.3:** Schematic diagram illustrating the QI mode available on the JPK NanoWizard 3 AFM. The tip is lowered into contact with the surface, a set force is rapidly loaded, and the tip is then retracted by a prescribed distance. After a short additional retraction, the tip is repositioned and the process is repeated. In this manner, a grid of force curves is created with topographical, stiffness and adhesion data available at each point.
1.4 AFM Measurements of the Mechanical Properties of Bacterial Cells

1.4.1 Early AFM measurements of mechanical properties

A crude characterization of the mechanical properties of bacterial cells using AFM can be obtained by measuring the force required to rupture the cell. Bolshakova et al. used this technique to compare the stiffness of the vegetative and mummy forms of *Arthrobacter globoformis* [29]. They scanned the two dormant forms of *A. globoformis* bacteria in force modulation mode at a set force of 1 µN. This force set point exceeded the imaging force set point by a factor of 100 and was sufficient to burst the cell wall of the mummy form of the bacteria, while the vegetative form remained intact [29].

A more quantitative measure of bacterial stiffness can be achieved by force indentation measurements. Arnoldi et al. demonstrated that force indentation curves can be used to obtain a measure of the Young’s modulus and turgor pressure of the cell [30]. Bailey et al. rapidly loaded 1 nN of force onto *Staphylococcus aureus* cells trapped in micron-sized wells etched into silicon and coated with polystyrene [31]. Using Arnoldi’s analysis, Bailey et al. measured a constant turgor pressure of *S. aureus* cells throughout the cell cycle. Longo et al. used a force mapping AFM mode to perform a series of force indentation curves along the surface of an *E. coli* bacterial cell [32]. By repeatedly imaging the cell, they were able to observe changes to the topology of the stiffness of the cell over time, illustrating the dynamic nature of the bacterial cell wall and underlying structures [32].

1.4.2 Mechanical Response to Antimicrobials

Force indentation measurements of live cells have been further used to identify the effects of pH [33], carbon nanotubes [34] and antimicrobial peptides [35], on the mechanical properties of cells. Rossetto et al. combined force indentation measurements and AFM imaging
of *P. aeruginosa* bacterial cells to gain insight into the mechanism of action of the novel antimicrobial peptide SB006 [36]. By providing evidence that the stiffness of the cell does not change after exposure to SB006 at bactericidal concentrations, Rossetto *et al.* showed that SB006 does not degrade the integrity of the peptidoglycan sacculus of the bacterial cell wall. They also observed the fragmentation of the outer bacterial membrane during antimicrobial treatment. These measurements support the proposed mechanism of action of the antimicrobial, in which SB006 binds to the outer phospholipid membrane [36].

1.5 Creep Deformation Experiment

Force indentation measurements measure the instantaneous elastic response of the cell to an applied force. Bacterial cells are complex soft materials that relax in a time dependent manner in response to an applied force. Additional insight into the mechanical properties of the cell may be obtained by quantifying the viscoelastic properties of the cells. Creep deformation is a common engineering technique that is used to evaluate the viscoelastic nature of a material; AFM can be used to perform creep measurements at the microscale on individual bacterial cells.

In the creep deformation experiment, the force is rapidly applied to the cell. Because the force loading time is short compared with the time-dependent mechanical response of the cell, the elastic response of the cell is probed. The corresponding spring constant $k_1$ of the bacterial cell can be obtained directly from the linear portion of the force-displacement curve during loading. After loading, the force is held constant for a period of time and the creep deformation of the cell is measured as the change in the indentation of the cell.
Figure 1.4: Schematic representation of the creep deformation experiment. During the force indentation of the bacteria, the AFM tip rapidly loads a set force that indents the cell. The creep deformation response is then measured as the cell continues to indent with time with the applied force held constant. The creep deformation of the cell as a function of the force dwell time is extracted from the z-piezo displacement data.
The AFM creep deformation experiment was developed by Vadillo-Rodriguez et al. [37-39], which allowed the measurement of the time dependent, viscoelastic response of bacterial cells to an applied force. The creep deformation technique was also applied to the study of the viscoelastic properties of bacterial biofilms [40, 41].

1.5.1 Viscoelastic Models for Creep Experiments

To quantify the viscoelastic nature of bacterial cells, the creep deformation data are interpreted in terms of viscoelastic models that describe the stress-strain relationship for complex materials. These models are constructed as combinations of two simple components: an elastic spring and a viscous dashpot. Dashpots can be conceptualized as a piston moving in a cylinder filled with a viscous fluid. Because of their viscous nature, dashpots have a time dependent response, obeying the relation $\sigma = \eta \frac{d\varepsilon}{dt}$, where $\sigma$ is the stress, $\frac{d\varepsilon}{dt}$ is the strain rate, and $\eta$ is the viscosity. In contrast, springs have an instantaneous, elastic response to an applied stress, with the stress-strain relationship described by the equation $\sigma = E\varepsilon$, where $E$ is Young’s modulus.

Different viscoelastic models are created by using different combinations of parallel and series arrangements of springs and dashpots, analogous to the modeling of electrical circuits using parallel and series combinations of resistors and capacitors. Springs and capacitors store energy introduced to the system, whereas dashpots and resistors dissipate energy.

The standard solid model, which consists of two springs and a dashpot, is shown schematically in Figure 1.5. This model corresponds to a time dependent displacement $Z(t)$ given by:

$$Z(t) = \frac{F_0}{k_1} + \frac{F_0}{k_2} \left(1 - e^{-(k_1/\eta_2)t}\right)$$  \hspace{1cm} (1.1)
The Burger’s model, which corresponds to the standard solid model in series with an additional dashpot, is shown schematically in Figure 1.5. This model corresponds to a time dependent displacement $Z(t)$ given by:

$$Z(t) = \frac{F_0}{k_1} + \frac{F_0}{k_2} \left(1 - e^{-(k_1/\eta_2)t}\right) + \frac{F_0}{\eta_1} \cdot t$$

(1.2)

At long times, the third term on the right hand side of Eq. 1.2 dominates, and the displacement increases linearly with time. For bacterial cells, this linear term corresponds to the compromising of the mechanical integrity of the bacterial cell envelope, and is specified by the permeability parameter $1/\eta_1$. The value of $k_1$ is related to the turgor pressure of the cell [30]. The parameters $k_2$ and $\eta_2$ correspond to the stiffness and viscosity of the periplasmic space respectively [42].

**Figure 1.5:** Schematic diagram of the viscoelastic models used in this study. The standard solid model (A) (adapted from [42]) was used to analyze the creep deformation data collected on cells
before exposure to antimicrobial peptides. The Burgers model (B) (adapted from [42]) was used to analyze data collected on cells after exposure to antimicrobial peptides.

1.5.2 Creep Deformation to Characterize Antimicrobial Activity and Action

Vadillo-Rodriguez et al. developed the AFM creep deformation experiment, and their measurements of individual Gram-negative and Gram-positive bacterial cells showed that the creep deformation was well described by the standard solid model of viscoelasticity [37-39].

Antimicrobials such as PMB, PMBN, and lysozyme damage components of the bacterial cell wall. Lu et al. used the AFM creep experiment to characterize the antimicrobial activity and action of PMB and PMBN. Exposure of *P. aeruginosa* PAO1 cells to PMB and PMBN resulted in measured creep deformations that were not well described by the standard solid model. Instead, these data were well described by the Burgers model. This was interpreted as being due to the formation of holes in the outer (PMB and PMBN) and inner (PMB) membranes of the cells, such that the force applied by the AFM tip caused leakage of material from the periplasm (PMB and PMBN) and cytoplasm (PMB) of the cell. By fitting the creep deformation data to Eq. 1.2, the changes to the viscoelastic parameters of the cells before and after exposure to PMB and PMBN were quantified. These results were consistent with the known mechanism of action of the antimicrobials, with the change in the permeability parameter $1/\eta_1$ twice as large for PMB as for PMBN. Changes to the viscoelastic parameters for individual bacterial cells were also tracked as a function of time. For each cell, a catastrophic event, consisting of a dramatic increase in the permeability parameter and corresponding changes in the other viscoelastic parameters, occurred after exposure to PMB or PMBN. The cells were compromised within a short period of time,
suggesting that a critical concentration of antimicrobial molecules is required to compromise the membrane [42].

The application of the viscoelastic models to bacterial cells is particularly powerful because it is possible to associate a physical effect or component with each of the viscoelastic parameters. As discussed above, the value of $1/\eta_1$ is a measure of the permeability of the cell envelope. The value of $k_1$ is related to the internal turgor pressure of the cell. Changes to the values of $k_2$ and $\eta_2$ upon exposure to the antimicrobials can be related to the dilution of the periplasm as molecules are removed and replaced by water [42].

The present work attempts to characterize the activity and action of PMB on *P. aeruginosa* bacterial cells in more physiological conditions. By performing these experiments in PBS buffer, the bacterial cells are not exposed to the large osmotic pressures induced by Milli-Q water environments. Validation of this experimental procedure will facilitate the study of a wide variety of bacteria and antimicrobials, involving antimicrobial mechanisms that depend on cellular activity that may be absent at larger osmotic pressures.
Chapter 2

Materials and Methods

2.1 Preparation of Bacterial Cells

Frozen stocks of *P. aeruginosa* PAO1 and *E. coli* K12 wild type cells were used to grow cultures on 2% w/v Lysogeny Broth (LB) agar (Fischer Scientific) plates. Single colonies were then selected and grown for 15-17 h overnight in an incubator at 37 °C and 150 rpm, in 50 mL of a 2% w/v LB (Fischer Scientific) solution. This growth time ensured that the cells were in the late exponential phase of the growth curve. Frozen stock was replenished by adding 500 µL of bacterial solution to 500 µL of 40% glycerol, vortexing the mixture and storing it in a -80 °C freezer. Samples were prepared by transferring 1 mL aliquots of bacterial solution into 1.5 mL centrifuge tubes, and centrifuging them for 3 min at 2200×g. After each centrifugation step, the supernatant was removed and the cells were re-suspended in Milli-Q water (resistivity of 18.2 MΩ·cm) or PBS buffer. This centrifugation and washing technique was performed three times for each aliquot. The bacterial solution was then diluted to 40% v/v by the addition of Milli-Q water to achieve an appropriate cell density for the AFM experiments.

2.2 Preparation of Glass Cover Slides Coated in Poly-L-Lysine

Glass slides were sonicated for 5 min in 2% RBS 35 (Thermo Scientific) and then rinsed with Milli-Q water, followed by a rinse with methanol or propanol, and rinsed once more with Milli-Q water. Slides were dried under nitrogen gas and a solution of 0.02% poly-L-lysine (Sigma-Aldrich) was Pasteur pipetted onto the slides until the surface of the slides was
completely covered. The solution was then left on the slides for 1.5 h at which point the slides were rinsed six times using Milli-Q water. Slides were then stored wet at 4°C for no more than three days prior to use.

2.3 Preparation of Glass Cover Slides Coated in Mussel Adhesive Protein

Glass slides were first acid cleaned for 2 h; they were then rinsed with water and dried under nitrogen gas. A solution was then prepared that consisted of 10 μL of Cell-Tak™ (a commercially available mussel adhesive protein), 285 μL of 0.1 M sodium bicarbonate and 5 μL of 1 M NaOH. Immediately after the solution was prepared, 60 μL of the solution was deposited into the center of each glass slide. The slides were then left in air for 20 min before being rinsed gently using Milli-Q water three times and stored wet at 4°C for no more than three days before use [43].

2.4 Immobilization of Bacterial Cells on Glass Cover Slips

Cells were immobilized on the surface of a glass microscope slide by depositing 1 mL of 40% v/v bacterial solution onto the area of the slide treated with either poly-L-lysine or MAP. The bacteria were allowed ~20 min to attach to the surface, and then the glass slides were rinsed using Milli-Q water or PBS buffer. This rinsing was performed to ensure the removal of any bacteria that were not firmly attached to the surface. The glass slide was then transferred to the AFM stage where the adhesion of bacterial cells could be observed by eye from a slight blue discoloration of the glass slide. After the AFM was properly calibrated, an AFM image of the glass slide was collected as detailed in section 2.5. This was done to check that the density of cells attached to the slide was appropriate for the study. If the density was too low, a second slide
was prepared using a longer adhesion time; alternatively, an undiluted aliquot of cells was used to ensure that a sufficiently large density of cells was obtained on the surface.

2.5 Imaging and Force Spectroscopy

AFM measurements were performed using an Asylum MFP-3D AFM (Asylum Research, Santa Barbara, CA) and a JPK NanoWizard 3 (JPK Instruments, Berlin, Germany). Contact mode and QI mode images of cells were collected using V-shaped silicon nitride cantilevers with a reflex gold coating, a sharp tip of radius < 10 nm, and a typical spring constant of 0.08 N/m (Nanoworld). Tapping mode images were collected using a stiffer 0.3 N/m cantilever with a resonance frequency of ~100 kHz in buffer. Force spectroscopy data was collected using a 1 μm diameter SiO$_2$ colloidal tip on a V-shaped silicon nitride cantilever with a reflex gold coating, and typical spring constants between 0.06 and 0.12 N/m (Novasco Technologics, Inc.). The spring constant of each new cantilever was characterized by immersing the cantilever in 1 mL of Milli-Q water and measuring its thermal fluctuations [44]. The tip was then used to collect a deflection-displacement curve on a clean glass slide [44]. After mounting the glass slide with bacteria immobilized on the surface in the AFM stage, 1 mL of solution (either Milli-Q water or PBS buffer) was pipetted onto the slide and the AFM was lowered onto the sample such that the cantilever was positioned inside the droplet.

The Asylum MFP-3D AFM was used in contact mode to image *P. aeruginosa* PAO1 bacterial cells, attached to a glass slide using poly-L-lysine (Sigma-Aldrich) in Milli-Q water. The cells were imaged by collecting 10 μm x 10 μm scans at a 256 x 128 pixel resolution, with a scan rate of 1 Hz and a force set point of ~1 nN.

To image bacterial cells in buffer conditions, *E. coli* K12 or *P. aeruginosa* PAO1 PilT bacteria in PBS buffer were deposited onto a glass coverslip coated with Cell-Tak™ and imaged
using the Asylum MFP-3D AFM AC mode or JPK NanoWizard 3 QI mode. Using the Asylum MFP-3D AFM in IC mode, a drive amplitude of \(~1.2\) V was used to achieve a tapping amplitude of \(1\) V at a frequency of \(~100\) kHz. An amplitude set point of 600 mV was used to image a 10 µm x 10 µm area using a scan rate of 1 Hz and a resolution of 256 x 128 pixels. For QI mode images collected using the JPK NanoWizard 3, a force set point of 10-25 nN was used with a \(z\)-piezo extend/retract speed of 25 µm/s, a Z length of 700 nm and an additional retract of 100 nm. An image of 128 x 128 pixel resolution was collected with a next line delay of 1000 ms.

To collect force spectroscopy data, the cells were rapidly loaded with a force of 6 nN at a rate of 1.98 µm/s. As the total indentation is of the order of tens of nanometers, the force was loaded on the order of 0.01 s. The force was then held constant for 6 s as the vertical displacement of the AFM cantilever was recorded. The further indentation of the cantilever into the bacterial cell corresponds to the creep deformation of the cell and this response can be used to calculate the viscoelastic parameters of the cell.

Force spectroscopy measurements on \(P.\ aeruginosa\) PAO1 pilT bacteria in PBS buffer were performed without AFM imaging using the Asylum MFP-3D in conjunction with a Nikon TE2000 inverted optical microscope. A 60x oil immersion objective was used to image bacteria attached to the surface with Cell-Tak™ and position the AFM cantilever above isolated cells. Force spectroscopy measurements were then performed using the Asylum MFP-3D AFM without the risk of dislodging the cells from the surface during contact mode or AC mode scans.

Stability measurements of the JPK NanoWizard 3 were conducted in air by rapidly loading a force of 6 nN to 20 nN onto a clean glass microscope slide 1 mm in thickness. The applied force was then held constant for between 6 s to 3 h and the height (measured) was recorded. The height (measured) is a measure of the change in the extension of the \(z\)-piezo
corrected for factors such as piezo hysteresis. A positive height (measured) corresponds to a retraction of the z-piezo in response to a measured increase in the deflection of the cantilever away from the surface. These measurements were combined with temperature measurements by placing a TSP01 compact thermistor sensor (Thor Labs) on the microscope slide 1 cm from the AFM head.

Stability measurements on the Asylum MFP-3D were conducted in PBS buffer by rapidly loading 6 nN of force onto a clean glass coverslip 0.17 mm in thickness. The applied force was then held constant for 6 s to 10 s. Z-piezo displacement is a measure of the change in the extension of the z-piezo required to maintain the force applied to the glass coverslip. Similar to height (measured), a positive z-piezo displacement results from the retraction of the z-piezo in response to a measured increase in the deflection of the cantilever away from the surface.

2.6 Addition of Cationic Peptides and Time Resolved Measurements

Either PMB (Sigma-Aldrich) or PMBN (Sigma-Aldrich) was dissolved in Milli-Q water or PBS buffer to a concentration of 1 mg/mL. Solutions of 1 mg/mL were stored at -20 °C before use. Solutions were thawed and diluted to 100 μg/mL immediately prior to use. For time resolved studies, a series of 10 μm × 10 μm scans was collected in a grid surrounding the cluster of cells of interest before the cells were exposed to the peptide. The topography images from each of the scans were saved and force spectroscopy data was collected by performing a creep deformation experiment near the center of a number of cells in each area. The AFM tip was then disengaged and raised slightly. Care was taken not to move the AFM and the sample, and 500 μL of water was removed from the fluid cell using a micropipette. 500 μL of the peptide solution diluted to a concentration of 100 μg/mL was then added to the fluid cell, such that the final concentration of the antimicrobial was 50. μg/mL, which was the value recommended by Sigma-
Aldrich for clinical use (more than 25 times the minimum inhibitory concentration). The AFM tip was then lowered carefully back into contact with the sample and several 10 μm × 10 μm scans were collected in the same area of the sample. By comparing the newly obtained topography scans to the previously saved images, it was possible to identify cells that had been measured before the introduction of the peptide. These cells were then repeatedly subjected to the creep deformation experiment, so that changes to the mechanical properties of the cells with time could be measured. Creep deformation measurements were also performed on a large number of bacterial cells before and at least 2 h after the introduction of the peptide to characterize the effect of the peptide on the mechanical properties of the cells.

2.7 Data Fitting Procedures

We used MATLAB® R2011a v. 7.12.0.635 to fit the data from creep deformation experiments to the Burgers model described in section 1.4.2 and specified by equation 1.2 with fitting parameters $k_2$, $\eta_2$, and $\frac{1}{\eta_1}$. To perform the fitting, the function lsqcurvefit() was used; lsqcurvefit() solves nonlinear data fitting problems using a least squares approach. This function determines the coefficients $x$ that solve the problem shown in equation 2.1.

$$\min_x \| F(x, xdata) - ydata \|^2 = \min_x \sum_i (F(x, xdata_i) - ydata_i)^2$$  \hspace{1cm} (2.1)

where $xdata$ is the given input data, $ydata$ is the observed output data, and $F(x,xdata)$ is the function.

We also used the lsqcurvefit() optional inputs for upper and lower bounds, and initial estimations of the coefficients to improve the convergence of the fitting procedure. The initial values of $k_2$, $\eta_2$, and $\frac{1}{\eta_1}$ were chosen to be 0.1, 0.1, and 0, respectively, such that they were close to the average values measured for an intact Gram-negative cell [8]. The lower bounds of $k_2$, $\eta_2$,
and \( \frac{1}{\eta_1} \) were chosen to be 0, 0, and -0.5 respectively. The lower bounds on \( k_2 \) and \( \eta_2 \) were chosen to prevent negative values for the best-fit values of the parameters, as these would not represent physically meaningful values. Although the theoretical lower limit for \( \frac{1}{\eta_1} \) is 0, some of the data sets yielded slightly negative values of \( \frac{1}{\eta_1} \) due to small amounts of thermal drift in the system and uncertainties associated with the fitting. The lower bound for \( \frac{1}{\eta_1} \) of -0.5 was chosen to allow the analysis of these data sets. The upper bounds of \( k_2, \eta_2, \) and \( \frac{1}{\eta_1} \) were chosen to be 3, 3, and 3, respectively, such that they were 50% larger than the maximum values of the coefficients that were previously measured in creep deformation experiments on \( P. \) aeruginosa cells. Additionally we used the function tolerance option, which terminated the fitting algorithm when the change in the best-fit values between iterations was less than the chosen tolerance (\( 10^{-16} \)).
Chapter 3

Results and Discussion

I will begin this chapter by providing an overview of my contributions to work published by Lu et al. [42]. These measurements were performed on \textit{P. aeruginosa} PAO1 bacterial cells in Milli-Q water using the Asylum MFP-3D AFM. To address concerns about the relatively high PMB concentration of 50. µg/mL used in ref. [42], I present measurements in Section 3.1 that I performed using a lower concentration of 5.0 µg/mL, which is less than 5 times the minimum inhibitory concentration of PMB. In Sections 3.2 and 3.3, I describe my attempts to characterize the effects of PMB on cells in phosphate-buffered saline (PBS) buffer. To further demonstrate the capacity of the creep deformation experiment to classify and characterize novel antimicrobials, the results of adapting the procedure to more native bacterial conditions are presented.

3.1 Measurements of \textit{P. aeruginosa} PAO1 Cells in Milli-Q Water with the Asylum MFP-3D

AFM topography images and creep deformation data were collected on \textit{P. aeruginosa} PAO1 bacterial cells before, during and after 2 h of exposure to 5.0 µg/mL of PMB for 2 h. In the first experiment, two bacterial cells were imaged repeatedly for 2 h following exposure to PMB. In the second experiment, creep deformation curves were collected from 70 bacterial cells before and after a 2 h exposure to PMB. In the third experiment, the creep deformation of a single bacterial cell was repeatedly measured (6 s per measurement) for 1 h after exposure to
PMB. The results presented in this section are in good agreement with the results observed at 50 \( \mu g/mL \) as reported by Lu et al. [42].

### 3.1.1 AFM Images of Cells Before and After Exposure to PMB

AFM images of two *P. aeruginosa* PAO1 bacterial cells were collected before exposure to PMB and then repeatedly collected for 2 h after exposure to 5.0 \( \mu g/mL \) of PMB. Representative AFM topography images are shown in Figure 3.1, and these images illustrate the effect of PMB on the outer surface of the cells. The image of Figure 3.1 a) was collected 119 minutes before exposure. Figure 3.1 b) was collected after an additional 114 min, and the image is nearly identical to that shown in Figure 3.1 a). This is reasonable since *P. aeruginosa* PAO1 cells are stable in Milli-Q water for long periods of time [38]. Figure 3.1 c) was collected 26 minutes after exposure to PMB, and shows an increase in surface roughness of the cells, indicating damage to the cell envelope. This is a direct result of exposure to the antimicrobial. Figures 3.1 d), e) and f) were collected 34, 67 and 102 minutes respectively after exposure to PMB and show a similar increase in surface roughness. The degradation of the cell membrane can be quantified by comparing the heights of the two cells before and after exposure. The heights of each cell decrease by \( \sim 15\% \) from 590 nm to 505 nm for the top cell and 610 nm to 530 nm for the bottom cell. The increase in cell surface roughness is due to the degradation of the outer and inner membranes by PMB, corresponding to the release of LPS molecules from the outer membrane and the release of molecules from within the periplasmic and cytoplasmic space [16].
Figure 3.1: Contact mode AFM topography images taken of *P. aeruginosa* PAO1 in Milli-Q water before and after exposure to 5.0 µg/mL of PMB. Image a) was collected 119 min before exposure to PMB; the cells have heights of 590 nm for the top cell and 610 nm for the bottom cell. Image b) was collected 5 min before exposure; the cells remain intact and the same shape and size. Image c) was collected after 26 min of exposure to PMB; the cell surface is rougher and the edges of the cells are jagged. Images d), e) and f) were collected 34, 67 and 102 min respectively after exposure to PMB. The outer surface of the cells is roughened in each of these images; the cells have heights of 505 nm for the top cell and 530 for the bottom cell. Figure adapted from reference [42].
3.1.2 Creep Deformation of Cells Exposed to PMB

A creep deformation curve from a *P. aeruginosa* PAO1 cell before exposure to PMB is shown in Figure 3.2 a). A 5 nm creep deformation occurred during the first 2 s of the measurement, followed by a gradual approach of the displacement of the cantilever to a plateau value of 5 nm. In contrast, a representative creep deformation curve from a *P. aeruginosa* PAO1 cell after exposure to 50. µg/mL of PMB for 2 h is shown in Figure 3.2 b). A 20 nm creep deformation occurs during the first 2 s of the measurement, followed by a linear increase in the displacement of the cantilever with time. The creep curve in Figure 3.2 b) can be modeled using the Burgers model (Section 1.5.1), as specified by Equation 1.2 and shown schematically in Figure 3.2 d). The second dashpot with viscosity $\eta_1$ shown in Figure 3.2 d) mathematically represents the linear term in Equation 1.2. The data collected on the untreated cell (Figure 3.2 a) can be fit using Equation 1.2 with $1/\eta_1 = 0$ (shown in Figure 3.2 c). The value of $1/\eta_1$ corresponds to the slope of the creep curve at long times and it is a direct indication of the compromising of the mechanical integrity of the bacterial cell envelope. As PMB forms pores in the outer and inner membranes, the constant applied force during the creep measurement causes the release of cellular material through the pores, producing a linear increase in the displacement of the AFM cantilever into the cell at long times. The values of $\eta_2$ and $k_2$ are related to the viscosity and stiffness of the periplasmic space [42]. As molecules are forced out of the periplasmic space during the creep deformation experiment, it becomes more water-like corresponding to a decrease in the best-fit $\eta_2$ and $k_2$ values and an increase in the magnitude of the creep deformation. The turgor pressure of the cell, which resists the indentation, gives rise to an instantaneous elastic response represented by the value of $k_1$, which is obtained from a fit to the force-indentation data measured during the force loading of the cell as described in Section
1.5. The damage to the cell membranes results in a decrease in the turgor pressure and a smaller best-fit value of $k_1$.

**Figure 3.2:** Representative creep deformation data collected on *P. aeruginosa* PAO1 cells (a) before and (b) after exposure to PMB. (c) A schematic diagram of the standard solid model and the equation for the deformation as a function of time. (d) A schematic diagram of the Burgers model and the equation for the deformation as a function of time. Figure adapted from reference [42].

Creep deformation measurements were performed on populations of *P. aeruginosa* PAO1 cells before and after 2 h of exposure to 5.0 µg/mL of PMB. The best-fit values of the four
viscoelastic parameters \(1/\eta_1, k_1, k_2\) and \(\eta_2\) were obtained by fitting the creep deformation curves for each cell to the Burgers model before and after exposure to PMB. The fits were performed using MATLAB routines as discussed in Section 2.6. Figure 3.3 contains histograms of the distributions of the best-fit viscoelastic parameters measured for 70 individual bacterial cells before and after exposure to 5.0 \(\mu\)g/mL of PMB.

The distribution of the best-fit values of the \(1/\eta_1\) parameter (Figure 3.3 d) before exposure to PMB is narrow and centered at 0, indicating that the creep deformation of the cells approaches a constant plateau value at long times. After exposure to PMB, the distribution of best-fit values of the \(1/\eta_1\) parameter (Figure 3.3 d) shifts to a positive value of \(\sim 0.02\) m/Ns, indicating that the creep deformation does not approach a plateau value at long times. This shift in the \(1/\eta_1\) parameter can be attributed to the permeabilization of the outer membrane [42]. PMB forms pores in the inner and outer membranes of cells that permeabilizes them and allows cellular material to leak out through the pores.

The average best-fit values of the parameters \(k_2\) and \(\eta_2\) both decrease significantly after exposure to PMB, as can be seen in the histograms presented in Figure 3.3 b) and c) respectively. This decrease is consistent with the periplasmic space of the cells becoming less viscoelastic and more water-like. These shifts of the parameters \(1/\eta_1, k_2\) and \(\eta_2\) are qualitatively consistent with creep measurements collected on \(P.\ aeruginosa\) PAO1 exposed to 50. \(\mu\)g/mL of PMB [42].

The average best-fit value of the parameter \(k_1\) decreases from approximately 0.4 N/m before exposure to PMB to approximately 0.2 N/m after exposure PMB, as shown in Figure 3.3 a). The decrease of \(k_1\) with exposure to PMB is consistent with a decrease in the turgor pressure of the cell due to the compromising of the mechanical integrity of the bacterial cell envelope. Measurements collected on \(P.\ aeruginosa\) PAO1 exposed to 50. \(\mu\)g/mL of PMB for 1 h indicate
the average value of $k_1$ increases from 0.15 N/m to 0.17 N/m. Concentrations of PMB over 20 μg/mL are known to depolarize the cell membrane; the depolarization of the membrane will result in the cessation of cellular metabolism. The loss of cellular metabolism is likely the cause of the increase in the measured value of $k_1$ after exposure to 50. μg/ml of PMB. As such, the observed decrease in $k_1$ at the lower concentration of 5.0 μg/mL is likely a result of a difference in the mechanism of action compared to cells exposed to 50. μg/mL PMB.

![Histograms of viscoelastic parameters](image)

**Figure 3.3:** Histograms of viscoelastic parameters (a) $k_1$, (b) $k_2$, (c) $\eta_2$ and (d) $1/\eta_1$ for *P. aeruginosa* PAO1 cells before (grey) and after (coloured) 2 h of exposure to 5.0 μg/mL of PMB.
The viscoelastic parameters are defined in Section 1.5.1. In each plot, the overlap between the two histograms is indicated by the darker colour. Figure adapted from reference [42].

3.1.3 Time-Resolved Response of Bacterial Cells to PMB

To further characterize the activity of PMB on *P. aeruginosa* PAO1 cells, the creep deformation experiment was performed repeatedly (10 s per measurement) on a single *P. aeruginosa* PAO1 cell after exposure to 5.0 µg/mL of PMB. The values of the viscoelastic parameters $1/\eta_1, k_1, k_2,$ and $\eta_2$ are plotted as a function of time after exposure to PMB in Figure 3.4. The values of $k_2, \eta_2$ and $1/\eta_1$ experience significant abrupt changes after approximately 10 min of exposure to PMB, as shown in Figures 3.4 b), c) and d). These rapid dramatic changes in the viscoelastic parameters indicate rapid dramatic changes in the mechanical integrity of the cell envelope. These changes are consistent with those measured by Lu et al. for a higher concentration (50. µg/mL) of PMB [42] and are consistent with changes in the AFM images of *P. aeruginosa* PAO1 cells before and after exposure to PMB (Figure 3.1). The dramatic and rapid degradation of the cellular envelope suggests a large number of pores were formed in the membrane simultaneously. This effect is best described by the accumulation of PMB on the bacterial cell surface and the rapid formation of pores once a critical concentration is reached [42]. In addition to this rapid change in viscoelastic parameters, there appears to be a slight recovery of the cell from 10 min to 60 min after exposure to PMB. At long experiment times, $k_2, \eta_2$ and $1/\eta_1$ appear to recover towards their initial values measured before exposure. The recovery of $1/\eta_1$ towards its initial value may be explained by the decreasing volume of the cell. As cellular material leaks through the permeabilized membrane, its volume decreases while the surface area of the cellular envelope remains constant. The resulting decrease in turgor pressure may be observed as a smaller leakage of material at long experimental times and a corresponding
recovery of the $1/\eta_1$ parameter. As the outer membrane becomes permeabilized and the material lost from the periplasm is replaced by water we observe a corresponding decrease in the $k_2$ and $\eta_2$ parameters. As such, the slight recovery observed in Figure 3.4 C) and D) may be a result of the permeabilization of the inner membrane and the periplasm becoming less water like as it is filled with cytosolic material.

**Figure 3.4**: Viscoelastic parameters for a representative *P. aeruginosa* PAO1 bacterial cell versus time of exposure to 5.0 g/mL PMB, which was introduced at time $t = 0$. (a), (b), (c) and (d) correspond to the parameters $k_1$, $k_2$, $\eta_2$ and $1/\eta_1$ versus exposure time respectively. Figure adapted from reference [42].
3.2 Measurements on *E. coli* cells in Phosphate-Buffered Saline Using the JPK Nanowizard 3

To perform AFM measurements under more physiologically relevant conditions, it is desirable to immerse the bacterial cells in PBS buffer rather than Milli-Q water. Milli-Q water increases the osmotic pressure of the cells and, for many bacterial strains, this causes the cells to burst. Despite this advantage of using PBS for the healthiness of the cells, charge screening due to the salts within the PBS buffer reduces the electrostatically attractive interaction between the negatively charged cells and the positively charged biological adhesive applied to the glass slide substrate. Therefore, to image the cells in PBS buffer, it is necessary to use a different adhesive that does not rely on electrostatic interactions. Many alternative adhesives, including mussel-adhesive protein (MAP) [45], provide a significantly weaker bond between the bacterial cells and the glass surface compared to e.g. poly-L-lysine (PLL). It is not possible to image bacterial cells adhered with MAP using contact or IC AFM modes, as the lateral forces applied to the cell during the rastering of the AFM cantilever during imaging dislodge the cells from the surface. This was observed in AFM images as a streaking across the imaged cells and cells being partially imaged before being dislodged from the surface. It is therefore appealing to try to use a different imaging modality that does not dislodge the cells from the surface during scanning.

3.2.1 QI Imaging of *E. coli* K12 Cells Using the JPK AFM System

The Quantitative Imaging (QI) mode of the JPK Nanowizard 3 AFM system (see Section 1.3.5) generates an image by collecting a dense 2D matrix of force-distance curves. Because the
AFM cantilever is retracted briefly between force-distance curves, the lateral forces applied to the sample by the AFM cantilever are very small. Using QI mode, *E. coli* K12 cells were successfully imaged in buffer, as shown in Figure 3.5. This image does not contain artefacts associated with removal of the cells from the substrate, e.g. streaks or half imaged bacteria, confirming that the QI mode applies lateral forces that are sufficiently small to avoid dislodging the cells in a buffer environment.

**Figure 3.5:** QI image of isolated *E. coli* cells in PBS buffer adhered to a 25 mm type 1 round glass coverslip with MAP, showing that the cells remain attached to the surface during imaging. Image was collected over 20 mins. The image corresponds to a 2D matrix of $256 \times 256$ force-distance curves. The white bar corresponds to a distance of 2 µm.
3.2.2 AFM Creep Measurements of *E. coli* K12 Cells on the JPK AFM System

Representative creep deformation curves measured on an *E. coli* K12 cell in PBS buffer and adhered with MAP to a 25 mm type 1 round glass coverslip are shown in Figure 3.6 in which the indentation (see Section 2.4) is plotted as a function of time. The total indentation is ~35 nm, which is similar to the indentation of creep curves collected on *P. aeruginosa* cells in Milli-Q water. However, the indentation occurs over a longer time scale (4 s compared to 2 s, for curves (a) to (e) in Figure 3.6). The creep curve in Figure 3.6 f) resembles the expected creep curve but displays a large amount of noise at long times. These results suggest that the JPK system is not sufficiently stable during the creep deformation measurement. To test the stability of the JPK system, we performed a series of tests.
Figure 3.6: A collection of representative creep deformation curves from an *E. coli* K12 cell in PBS buffer adhered with MAP to a 25 mm type 1 round glass coverslip. a) through e) The curves
approach their maximum indentation depth over 3 s to 4 s, which is a larger time interval than the 2 s time interval to reach maximum indentation observed for *P. aeruginosa* PAO1 cells in Milli-Q water (Figure 3.1). As well, the indentation for the *E. coli* K12 cells does not plateau at long times. For this curve, the maximum indentation is achieved after 2 s of creep reaching a constant plateau value at longer times. However, there is a significant amount of drift and noise in the curve.

### 3.2.3 Measurements of the Stability of the JPK AFM System

To evaluate the stability of the JPK NanoWizard 3 AFM system, the change in the height (measured) (see Section 2.4) was measured as a function of time on bare glass microscope slides (thickness of 1 mm) in pure Milli-Q water. Of course, the rigid glass slide should not experience any measurable creep deformation in response to the force (several nN) applied by the AFM cantilever. Before each measurement, the JPK system was allowed to stabilize inside the enclosure for a period of at least 60 min. A representative curve of the height (measured) as a function of time is shown in Figure 3.7, which contains the essential features of all measured curves: an initial overshoot (~ 4 nm for the data in Figure 3.7) just after \( t = 0 \) s, followed by a non-monotonic drift (~7 nm for the data in Figure 3.7). Given the overshoot and non-monotonic character of the curves measured on a rigid substrate, it is clear that the measurement of the creep deformation of the bacterial cell is not possible. To investigate the origin of this undesirable system response, we performed a series of experiments.
**Figure 3.7:** Representative creep deformation curve collected on a bare glass microscope slide (thickness of 1 mm) in Milli-Q water using the JPK AFM system. The applied force was 6 nN. At the beginning of the curve (just after $t = 0$ s), there is a measurable overshoot, followed by a non-monotonic change in the height (measured) of ~7 nm.

To characterize the effect of thermal drift on the height (measured) with the JPK AFM system, the displacement of the $z$-piezo and the temperature near the scanner head were measured over an extended period of time following the closing of the acoustic enclosure box enclosing the AFM head. For these measurements, the AFM cantilever was brought into contact with a bare glass substrate. The temperature was measured using a Thor Labs TSP01 temperature
sensor. We found that changes in the height (measured) were generally correlated with changes in temperature, with an overall increase in both quantities with time. As shown in Figure 3.8, the system approaches thermal equilibrium rapidly in the first hour. Over the remaining 2.5 h, the temperature changes gradually by 0.4 °C. As such, the system can be considered to be near thermal equilibrium after 1 h.

![Temperature and Height Graph](image)

**Figure 3.8**: Temperature and height (measured) as a function of time for an AFM tip in contact with a clean glass microscope slide (thickness of 1 mm) in air with an applied force of 6 nN. The temperature rises by 2.5 °C in the first hour and then by only 0.4 °C over the next 2.5 h. The height (measured) increases approximately linearly with time by 250 nm over 3 h.
To characterize the effects of the small temperature change after the system has approached thermal equilibrium, the temperature and height (measured) were recorded over a 10-min period after the system had equilibrated for 3 h. Figure 3.9 shows that a temperature change of 0.12 °C was observed with a corresponding change in the height (measured) of 25 nm. As in Figure 3.8, the height measured was observed to increase with the temperature. However, as in Figure 3.8, changes in height (measured) over short times are not necessarily correlated with increases in temperature, e.g. near 400 s in Figure 3.9. This suggests that other factors may be contributing to changes in height (measured) with time.

**Figure 3.9:** Temperature and height (measured) as a function of time for an AFM tip in contact with a bare glass microscope slide (thickness of 1 mm) in air with an applied force of 6 nN. A
A small temperature change of 0.12 °C was observed during the 10 min duration of the experiment, together with a large 25 nm increase in the height (measured).

To further analyze the effect of small temperature changes on the height (measured), measurements over shorter time scales were performed after the temperature of the system was allowed to equilibrate for more than 3.5 h. In Figure 3.10 we show a representative creep deformation curve collected for 20 s on a bare glass surface using an applied force of 6 nN. The temperature change during this short time is of the order of 0.01 °C. Despite this small change in temperature, the height (measured) data in Figure 3.10 shows a large drift of 4 nm. The presence of the 4 nm drift indicates that factors other than thermal drift are responsible for the drift of the height (measured) with time. A drift of this magnitude would have minimal effect on the imaging of bacterial cells but would contribute a significant convolution to the creep-deformation measurements which measure deformations on the order of ~20 nm.
**Figure 3.10:** Temperature and height (measured) as a function of time for an AFM tip in contact with a clean glass microscope slide (thickness of 1 mm) in air with an applied force of 6 nN. During the short duration of this experiment, a large drift of 4 nm was observed with a corresponding small change in temperature of 0.01 °C.

In Figure 3.11 we show a representative creep deformation curve collected on a bare glass microscope slide with an applied force of 6 nN after an extensive effort to isolate the AFM from all external sources of noise. This included adjusting the cables leading to the AFM head to avoid vibration transmission, as well as modifying the camera stand to separate it from the acoustic isolation box. Additionally, foam padding was added under the door of the isolation box and into the cable outlet of the box to block these openings. The AFM was repositioned to
remove any tension on the cables and a hollow plastic cylindrical sample holder was epoxied to the glass microscope slide to limit the effect on the sample of air currents within the isolation box. In the curve shown in Figure 3.11, an overshoot was observed at very small times, as in Figure 3.7, and after ~2 s, a very low level of noise in the height (measured) (< 1 nm) was observed with no significant systematic drift. This is an indication that the AFM head had been very well isolated from external noise sources. However, the overshoot at short times followed by a drift in excess of 1 nm from 0 s to 2 s persists, which is likely due to the instrumental response of the AFM system to rapidly loading the force to a set point value. These features were present in each curve collected under these conditions. Following discussions with JPK Instruments, it is likely that these undesirable features are due to the force ramp algorithm implemented by JPK. This issue is under further investigation by JPK in the laboratories in Berlin. Unfortunately, these issues prevent the collection of reproducible and reliable creep deformation measurements on bacterial cells using this AFM system, and this motivated us to return to the Asylum MFP-3D AFM system until the issues with the force ramp algorithm are rectified by JPK.
Figure 3.11: Creep deformation curve collected on a clean glass microscope slide (thickness of 1 mm) in Milli-Q water. The noise observed after 2 s is rather low (~0.8 nm total drift). However, there is a significant overshoot in the height (measured) at the start of the curve and a significant drift (~ 1 nm) during the first 2 s of the experiment.

3.3 Measurements on *P. aeruginosa* PAO1 PilT cells in PBS buffer with the Asylum MFP-3D AFM System
3.3.1 Stability of the Asylum MFP-3D AFM System

To check that the Asylum MFP-3D AFM head is adequately isolated from environmental noise, the \( z \)-piezo displacement (see Section 2.4) was recorded as a function of time with the AFM tip in contact with a thick glass microscope slide (thickness of 1 mm) in Milli-Q water using an applied force of 6 nN. The results are shown in Figure 3.12, indicating that there is minimal drift and noise of \( \sim 1 \) nm during the 6 s duration of the experiment. The noise which is of magnitude of \( \sim 1 \) nm is comparable to the noise observed on the JPK. The drift observed on the Asylum is an order of magnitude lower than that observed on the JPK and is optimal for creep deformation experiments.
Figure 3.12: Creep deformation curve collected on a clean glass microscope slide (thickness of 1 mm) in Milli-Q water. The amplitude of the noise is \(~1\) nm during the 6 s experiment, with no significant drift in the z-piezo displacement.

3.3.2 Optical imaging of \textit{P. aeruginosa} PilT cells with the Nikon TE2000 Optical Microscope

To eliminate the need to visualize the cells by collecting AFM images, which could dislodge them from the underlying substrate, the bacterial cells were adhered to a thin glass coverslip using a thin coating of MAP, immersed in PBS, and optical images were recorded using a Nikon TE2000 optical microscope. The Asylum MFP-3D AFM is mounted onto a plate on top of the optical microscope, allowing optical and atomic force microscopy measurements to be made simultaneously. All subsequent experiments using the Asylum MFP-3D AFM were performed using 60 mm x 24 mm type 1 glass coverslips (hereafter referred to as glass coverslips, thickness of 0.17 mm). In addition, we used the PilT mutant of \textit{P. aeruginosa} (hyperflagellated mutant) to avoid twitching of the cells on the glass surface. In Figure 3.13 we show an optical image of a sample with an AFM tip in contact with the glass surface in the presence of bacterial cells in PBS. The AFM tip is visible in Figure 3.13 as the high intensity spot near the center of the image, and the bacterial cells can be observed as small objects throughout the image. The microscope is configured with a built-in AG Heinze HBO103 mercury lamp designed for use in fluorescence microscopy. The light intensity, heat generated by the bulb and significant warm up time of the mercury lamp is unsuitable for bright-field microscopy. Because of this, experiments were performed using an external illumination source. By translating the optical microscope stage, it is possible to position the tip over a bacterial cell and then to perform
a creep deformation measurement on that cell. However, the low level of illumination and the small AFM tip radius make it difficult to ensure that the tip is properly positioned over the centre of the bacterial cell. The bacterial cells are not visible while the cantilever is positioned above them, so it is necessary to bring the tip from the top of the image to a position above a cell. In this manner the colloidal probe, which is often visible in the image as seen in figure 3.13, can be positioned over an isolated bacterial cell.

![Image](image.png)

**Figure 3.13**: Optical image of *P. aeruginosa* PAO1 PilT cells on a glass coverslip in PBS buffer. The image was collected using a 60× oil immersion objective and a 1.5× magnification lens. The dark triangle in the middle of the image is the AFM cantilever that is withdrawn from the glass surface; near the end of this cantilever is a 6 µm diameter colloidal probe that is indicated by the red arrow. The image corresponds to an area on the sample that is 90 µm × 90 µm.
3.3.3 Creep Measurements on *P. aeruginosa* PAO1 PilT Bacterial Cells Using the Asylum MFP-3D AFM System

A representative creep deformation curve for a PilT bacterial cell in PBS buffer on a 60 mm x 24 mm type 1 glass coverslip measured on the Asylum MFP-3D AFM system using a colloidal probe cantilever and an applied force of 6 nN is shown in Figure 3.14. Almost all of the displacement of the cantilever occurs in the first 3 s, with the displacement reaching a plateau value at long times. This curve is consistent with previous creep deformation measurements on *P. aeruginosa* PAO1 in Milli-Q water [42].
Figure 3.14: Creep deformation curve of a *P. aeruginosa* PAO1 PilT cell on a 60 mm x 24 mm type 1 glass coverslip in PBS buffer using a colloidal AFM tip and an applied force of 6 nN. The creep deformation reaches a plateau value at long times.

Unfortunately, creep deformation curves collected using the procedure described above were not reproducible. Approximately 40% of creep curves collected were similar to that shown in Figure 3.14. The remainder of the creep deformation measurements showed large negative decreases in the indentation, as shown in Figure 3.15. In Figure 3.15 A), we show a creep deformation curve with a relatively small drift away from the cell surface at long times. Approximately 40% of the measured curves contained a drift between 5 nm to 10 nm at long times, and nearly 20% of collected curves were qualitatively similar to that shown in Figure 3.15 B) in which the drift at long times is between 15 nm to 20 nm. We ascribe the large negative change in the indentation at long times to the bending of the thin glass coverslips in response to the force applied by the AFM cantilever.
Figure 3.15: Creep deformation curves of *P. aeruginosa* PAO1 PilT cells on a glass coverslip in PBS buffer using a colloidal AFM tip and an applied force of 6 nN. In a) and b), the curves have a significant negative slope at long times and do not reach constant plateau values. a) The drift is relatively small (5 nm) at long times. b) The drift dominates the creep deformation curve at long times, drifting away from the surface of the cell by nearly 20 nm.

With the MFP-3D AFM head mounted on top of the Nikon TE2000 optical microscope, it is possible to do AFM and optical microscopy measurements simultaneously. This configuration has the distinct advantage of allowing the positioning of the AFM tip over a bacterial cell for the collection of creep deformation curves without the need for scanning the AFM tip across the sample. However, there is an associated disadvantage for this configuration – the sample must be prepared on a thin glass coverslip, as required by the use of the oil immersion microscope objective. The use of thin coverslips complicated the creep deformation experiment because of the deformability of the coverslips in response to the force applied by the AFM cantilever. To evaluate how the bending of the coverslip affected the creep deformation experiments, the *z*-piezo displacement was measured as a function of time on a thin glass coverslip supported at its center by the 60x oil immersion objective. The *z*-piezo displacement channel is similar to that of the height (measured) channel on the JPK and is a measure of the height of the tip above the surface. Figure 3.15 shows a representative curve. A positive slope in the position of the *z*-piezo over time corresponds to a retraction of the *z*-piezo away from the surface of the coverslip. This result is qualitatively similar to that observed in Figure 3.7 at long times.
Figure 3.16: Z-piezo displacement as a function of time on a glass coverslip in PBS buffer using a colloidal AFM tip and an applied force of 6 nN. The z-piezo displacement increases by ~35 nm in 6 s.

In the experiment, the coverslip is placed across a 4 cm dia hole in the mounting plate for the AFM head, with the oil immersion microscope objective lens brought into contact with the lower side of the coverslip through the hole in the mounting plate. Because the hole in the mounting plate is so large, the thin glass coverslip can vibrate and flex in response to a force applied by the AFM cantilever and this is very detrimental to collecting high quality creep.
deformation curves. We attribute the large positive drift in the $z$-piezo displacement with time in Figure 3.15 as being due to the flexing of the glass coverslip. To try to reduce the flexing of the coverslip, we designed and constructed a series of aluminum inserts with central holes of different diameter (ranging from 1.25 cm dia to 1.75 cm dia). The inserts fit into the 4 cm dia hole in the mounting plate, reducing the unsupported area of the coverslip while still allowing full contact between the coverslip and the oil immersion lens. A schematic of the experimental setup with the metallic insert is depicted as below in Figure 3.17.

![Figure 3.17: A schematic diagram of the experimental setup with the metallic insert. The AFM head is shown in orange and the stage is shown in blue. The metallic insert (dark grey) is constructed and positioned such that it fits over the oil immersion objective without making contact, and is supported at the edges by the AFM stage.](image-url)
This should reduce the flexing of the coverslip during the creep deformation experiment. In Figure 3.18 we show a measurement of the z-piezo displacement with time collected on a glass coverslip using the insert with a 1.5 cm dia hole, with the 60x oil objective in contact with the underside of the coverslip. The use of the insert decreased the drift from 30 nm over 6 s (Figure 3.16) to 12 nm over 6 s (Figure 3.18). Despite the reduction in the drift with the use of the insert, it was still too large to accurately measure the creep deformation of bacterial cells, which typically has a total creep deformation of ~20 nm. Similar drifts with time were observed for the other aluminum inserts. With each insert, the drift ranged from 12 nm to 35 nm with no strong correlation between the magnitude of the drift and the width of the hole in the insert. However the range of hole diameters examined was only 0.5 cm (between 1.25 cm and 1.75 cm in diameter). To further test the effect of hole diameter on the magnitude of the drift, a wider range of hole widths in the inserts should be examined.

In addition, we tested the effect of allowing more time for thermal equilibration of the system before collecting the creep deformation curves. In Figure 3.18 we show a curve that was collected after a longer time (2 h) of thermal equilibration, and the drift was not significantly reduced from the previous measurements. This suggests that the observed drift is due to the unwanted flexing of the thin coverslip that is required for simultaneous use of the AFM and optical microscope.

Based on the results of all of the above experiments, we concluded that it was not possible to perform accurate measurements of the creep deformation of bacterial cells using the Asylum MFP-3D AFM/Nikon TE2000 configuration. The best path forward for performing creep deformation experiments on bacterial cells in PBS buffer is to try to improve the
performance of the JPK Nanowizard 3 AFM system such that accurate creep deformation experiments can be performed. In particular, we will work with JPK to see if it is possible to adjust the force loading parameters within the JPK software to prevent overshoot during force loading and make further improvements in environmental control to reduce the drift during the creep deformation experiments.

**Figure 3.18**: Creep deformation curve on a glass coverslip in PBS buffer using a colloidal AFM tip and an applied force of 6 nN, with the aluminum insert containing a 1.5 cm dia central hole. The $z$-piezo displacement increases by ~ 10 nm in 6 s.
Chapter 4

Summary and Conclusions

5.1 Summary of Results

We have developed an experimental procedure based on atomic force microscopy (AFM) to quantitatively study the action and activity of antimicrobial peptides. We have observed that the sensitivity of the creep deformation experiment is sufficient to measure the effects of clinical concentrations of PMB and PMBN on bacterial cells. We have extended these measurements to more biologically relevant conditions by performing them in buffer solutions. This has proved to be challenging and has revealed the limitations of performing the experiment on two different AFMs.

Specifically, we have utilized AFM to perform creep deformation experiments on individual bacterial cells to measure the deformation of the cells as a function of time while the cell is under a constant force. We find that the creep deformation curves can be fit to the Burgers model, allowing the characterization of the effect of antimicrobials on the bacterial cell wall in terms of changes in the viscoelastic parameters. The viscoelastic parameter $1/\eta_1$, which corresponds to a measure of the permeability of the inner and outer membranes, was observed to increase from approximately 0 m/Ns before exposure to PMB to an average value of about 0.1 m/Ns after exposure. This result, coupled with an observed decrease in both $k_2$ and $\eta_2$, support the known mechanism of action of PMB and validate our procedure as a means to characterize the effect of these antimicrobials. The analysis of the viscoelastic parameters of a single cell as a function of exposure time to PMB reveals a rapid change of membrane integrity after 10 min of
exposure. This result suggests the existence of a critical concentration on the surface of the bacterial cells for the antimicrobial action to occur.

In studies conducted on *P. aeruginosa* Pil T bacterial mutants in PBS buffer using the JPK NanoWizard 3, we observe a convolution of the bacterial creep with a time dependent response of the AFM z-piezo. We find the effects of the z-piezo movement immediately after force loading are sufficiently large to prevent the fitting of the collected data to the Burgers model. The effects of the z-piezo drift must be significantly reduced before meaningful creep deformation results can be collected using this AFM system.

We found that an Asylum MFP-3D AFM may be used with an inverted Olympus optical microscope to perform the creep deformation experiments on cells in buffer conditions. However, we observed a significant amount of drift during the creep deformation measurements that we attribute to the flexing of the glass coverslip: a large drift of approximately 30 nm is observed on a clean glass coverslip over a 6 s period. Supporting the glass coverslip with an aluminum insert reduced the observed drift to approximately 10 nm over the experiment duration, supporting the hypothesis that the drift was due to the flexing of the coverslip. However, this level of drift during the time scale of the creep deformation was still too large to permit meaningful creep deformation measurements on bacterial cells in buffer.

### 5.2 Future Work

The results of this thesis led to the development of an experimental methodology that allows the assessment of the action and activity of antimicrobial peptides. The limitations of this procedure arise from instabilities in two different AFM systems. The solution to this problem for the JPK AFM will be to collaborate with JPK Instruments to improve their force ramp algorithm.
such that overshoot and drift of the z-piezo is reduced. If the effects of the z-piezo drift could be significantly reduced, it should be possible to perform the creep deformation under biologically relevant conditions.

It would also be beneficial to study a wider variety of antimicrobials that affect different structures within the bacterial cell envelope. The AFM creep deformation experiment can contribute to the identification of the mechanism of action and be used to compare the effectiveness of antimicrobials that target cellular structures. For example, antimicrobials such as the glycopeptide vancomycin affect the integrity of the peptidoglycan layer, and understanding how degradation of this layer contributes to the creep deformation data would provide new insight into the action of this class of antimicrobials. Further development of this experimental procedure could lead to a powerful tool in the development of novel antimicrobials.
References


