Nanomechanical response of bacterial cells to cationic antimicrobial peptides†

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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 envelope, and our study introduces an AFM-based creep deformation technique to evaluate changes in the time-dependent mechanical properties of Pseudomonas aeruginosa PAO1 bacterial cells upon exposure to two different but structurally related antimicrobial peptides. We observed a distinctive signature for the loss of integrity of the bacterial cell envelope following exposure to the peptides. Measurements performed before and after exposure, as well as time-resolved measurements and those performed at different concentrations, revealed large changes to the viscoelastic parameters that are consistent with differences in the membrane permeabilizing effects of the peptides. The AFM creep deformation measurement provides new, unique insight into the kinetics and mechanism of action of antimicrobial peptides on bacteria.

Introduction

The cell envelope of Gram-negative bacteria is a dynamic, multilayered structure that consists of an outer membrane (OM) and a cytoplasmic membrane (CM), with an intervening thin periplasm containing the peptidoglycan sacculus. The cell envelope fulfills many crucial roles: it maintains the cell shape, resists internal turgor pressure, and selectively transfers molecules in and out of the cell, while allowing the cell to grow and divide. All of these functions rely on maintaining the mechanical integrity of the cell envelope and, because of this, many antimicrobial compounds target specific components of the cell envelope.

Despite great advances in the control of bacterial infections by using natural and synthetic antimicrobials, many bacteria have adapted to overused antimicrobials, developing resistance that renders the compounds ineffective. This adaptability of bacteria poses a serious threat to humans as antimicrobial-resistant “superbugs” emerge. There is a pressing need to develop novel antimicrobials such as cationic antimicrobial peptides (CAPs), as well as to obtain an in-depth understanding of their mechanism of action.

Although molecular-level descriptions of the mechanism of action of CAPs on bacterial cells are lacking, it is generally recognized that they bind electrostatically to the anionic cell surface. Many CAPs achieve their antimicrobial action by permeabilizing the cell membranes, however other CAPs translocate across the CM where they disrupt intracellular processes. The permeabilization of cell membranes by CAPs has been described in a number of models. 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.

In the present study, we have measured the effect of two structurally related CAPs, polymyxin B (PMB) and polymyxin B nonapeptide (PMBN), on the mechanical properties of Gram-negative Pseudomonas aeruginosa cells, an opportunistic human pathogen. PMB is a classic antimicrobial produced by Gram-positive Bacillus polymyxxa to out-compete neighboring Gram-negative bacteria. Clinical use of PMB is limited because it has high nephrotoxicity. Structurally, PMB is a pentacationic (charge of +5) lipodecapeptide consisting of a cyclic ring (dia 1.25 nm) and a fatty acyl tail. Because of its high positive charge, it binds to the negatively charged lipid A head group of lipopolysaccharide (LPS), displacing divalent cations that normally bridge the LPS molecules. By inserting its fatty acyl chain into the OM, PMB can pass into the periplasm through a self-promoted uptake mechanism, which also displaces LPS from the OM, forming holes in the membrane. Subsequently, PMB can disrupt the integrity of the CM.

PMBN is structurally very similar to PMB, as it is derived from PMB by enzymatic removal of the fatty acyl tail. It also binds to the LPS lipid A head group because of its high positive charge (charge of +4), releasing LPS from the OM and causing OM permeability. However, PMBN does not disrupt the CM.

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and, because of this, has much less antimicrobial activity than PMB.\textsuperscript{18,22} Instead, PMBN is used to sensitize bacterial cells to other antimicrobials, serving as an OM permeabilizer.\textsuperscript{20,23}

Protein release studies\textsuperscript{24} revealed that exposure of \textit{E. coli} K12 cells to 5 µg mL\textsuperscript{-1} PMBN resulted in the release of periplasmic proteins, indicating that PMBN only compromises the OM. In contrast, exposure to 5 µg mL\textsuperscript{-1} PMB releases both periplasmic and cytoplasmic proteins, indicating that PMB compromises both cell membranes. The time scale of action for both compounds was measured to be ~10 min, but the time resolution of this measurement was limited to ~5 min.

Atomic force microscopy (AFM) has been used to measure the elastic stiffness of bacterial cells\textsuperscript{26–29} by pressing the AFM tip into the cells and measuring their deformation. The effective spring constant measured from the linear part of the force–indentation curves is determined primarily by the internal turgor pressure,\textsuperscript{30} but also by the peptidoglycan layer and the MreB cytoskeleton.\textsuperscript{29,31} Recent studies have focused on changes to the elastic stiffness of bacterial cells with exposure to antimicrobials.\textsuperscript{32–36} Large variations in these results indicate the need for more detailed mechanical property information.

Recently, we developed a novel AFM-based technique to measure the time-dependent mechanical response of bacterial cells to a constant force applied by an AFM tip,\textsuperscript{37–40} which is the nanoscale equivalent of a creep deformation experiment. The experimental results were interpreted in terms of a simple arrangement of two springs and a dashpot known as the standard solid model.\textsuperscript{41} We demonstrated that the creep deformation experiment can be used to provide a robust, reproducible measure of the viscoelastic properties of bacterial cells. We observed significant differences between the viscoelastic properties of Gram-negative and Gram-positive cells,\textsuperscript{37,38} and we identified the mechanical role of LPP, the major peptidoglycan-associated lipoprotein in \textit{E. coli},\textsuperscript{39} as well as the effects of dynamic viscoelasticity.\textsuperscript{42}

In the present study, we used the creep deformation experiment to measure the effects of exposure to PMB and PMBN on the viscoelastic properties of \textit{P. aeruginosa} PAO1 bacterial cells. The peptides produced a distinctive signature for the loss of mechanical integrity of the cell envelope, and differences in both the magnitude and time scale of changes in the viscoelastic properties. By comparing the viscoelastic properties before and after 1 h of exposure, as well as performing time-resolved measurements after exposure, we obtained detailed information on four viscoelastic parameters that we can attribute to different components of the bacterial cell. We observed quantitative differences in the cellular response to the two peptides that are consistent with the difference in their membrane permeabilizing effects. In addition, we observed differences between exposure to high and low concentrations of PMB, which can be attributed to the depolarization of the CM, \textit{i.e.} a different mechanism of action, at the high concentration. Collectively, our results show that our AFM-based creep deformation technique provides unique information that is crucial for identifying the mechanism of action of antimicrobials.

### Experimental

#### Preparation of bacterial cells

\textit{Pseudomonas aeruginosa} PAO1 wild type and \textit{wapR} mutant bacterial cells were cultured on an LB agar plate from frozen stock. Bacteria were then grown overnight for 15–16 h in 50 mL LB growth medium at 37 °C to the late-exponential phase from a single colony on the LB agar plate. Bacterial cells were harvested by centrifugation (2200 \( \times \) g for 5 min), washed twice with ultrapure Milli-Q water (resistivity of 18.2 MΩ cm), and re-suspended in water (note that only ultrapure Milli-Q water was used in the present study). The centrifugation acceleration value used in the present study was significantly less than the value of 5000 \( \times \) g that was found by Pembrey \textit{et al.} to maintain the structural integrity of bacterial cell surfaces.\textsuperscript{43}

To immobilize the bacterial cells on the glass substrate, glass slides were coated with either poly-L-lysine (Sigma, part number P8920) or polyphenolic mussel adhesive protein (MAP) from the mussel \textit{Mytilus edulis} (Cell-Tak\textsuperscript{TM}, BD Diagnostics). Prior to the modification with poly-L-lysine, the glass slides were cleaned by sonication for 5 min in 2% RBS35 surfactant solution (Thermo Scientific, part number 27950), rinsed thoroughly with water, then with methanol, and then again with water.\textsuperscript{41} After being dried in air for 20 min, the glass slides were covered with 0.02% (w/v) poly-L-lysine for 2 h and rinsed with water again. The glass slides were kept wet at 4 °C for up to three days and air-dried immediately before the immobilization of the bacteria. Prior to modification with MAP, the glass slides were acid-cleaned for 2 h, rinsed with water, and air-dried. The central 1 cm\textsuperscript{2} area of each glass side was treated with 60 µL of a solution of 10 µL Cell-Tak\textsuperscript{TM}, 285 µL 0.1 M sodium bicarbonate and 5 µL 1 M NaOH immediately after the solution was prepared.\textsuperscript{44} The slides were left in air for 20 min and rinsed with water. They were kept wet at 4 °C for up to three days and air-dried immediately before the immobilization of the bacteria.

The bacterial cells were immobilized on the coated glass slides using the following procedure. 1 mL of the re-suspension of the bacterial cells was diluted by a factor of five with water, and deposited onto a glass slide coated with either poly-L-lysine or MAP. After 5–10 min, the bacterium-coated glass substrate was then gently rinsed with water three times to remove excess and loosely attached bacterial cells. The glass slide with immobilized bacterial cells was then transferred either to the AFM fluid cell for imaging and force measurements in liquid, or to the flow cell for optical microscopy.

#### AFM imaging and force measurements

Both AFM imaging and force measurements were performed using an Asylum MFP-3D AFM (Asylum Research, Santa Barbara, CA) on bacterial cells immobilized on glass substrates coated with either poly-L-lysine or MAP and placed in the AFM fluid cell. Three different types of AFM probes were used in our experiments: V-shaped silicon nitride cantilevers with a sharp tip (tip radius <10 nm, spring constant of 0.08 N m\textsuperscript{-1}, PNP-TR, Nanoworld), V-shaped silicon nitride tipless cantilevers with a 600 nm dia SiO\textsubscript{2} colloidal tip (spring constant of 0.06 or
0.12 N m \(^{-1}\), Novascan Technologies, Inc.), V-shaped silicon nitride cantilevers with a 1 μm dia SiO\(_2\) colloidal tip (spring constant of 0.12 N m \(^{-1}\), Novascan Technologies, Inc.).

The sharp AFM tips were used to collect high-resolution images of bacterial surfaces to observe the effects of exposure to PMB and PMBN on the morphology of the bacterial cells. AFM images were collected in contact mode using a low value of the applied force (~1–2 nN) and a scan rate of 1 Hz.

The colloidal AFM tips were used for both force and height measurements of the bacterial cells. We have carefully chosen the size of the colloidal particle on the AFM cantilevers (radius of 300 nm) such that is large enough to avoid nonlinear elastic effects in our mechanical measurements,\(^{27}\) and small enough to allow (low-resolution) imaging of cells for the purpose of performing the creep deformation measurement on isolated cells as a function of time. Initially, AFM images were collected to identify isolated, immobilized bacterial cells for subsequent study. Prior to each set of force measurements, the deflection sensitivity of the AFM cantilever was determined on a clean glass slide by collecting a force-displacement curve, and its spring constant was measured using the thermal fluctuation method within the AFM software package. The AFM force experiments consisted of (1) rapidly loading the bacterial cell at a rate of 1.98 μm s \(^{-1}\) to a preset value of the applied \(F_0\) (force-indentation), followed by (2) holding the value of \(F_0\) constant and measuring the displacement of the AFM tip (creep deformation). Force measurements were either performed before and after more than 1 h of exposure to PMB and PMBN, or as a function of time of exposure to PMB and PMBN. Bacterial cells were also imaged rapidly (1 image per min) to measure the time dependence of changes to the cell height caused by exposure to the peptides.

**Introduction of cationic peptides**

Either polymyxin B (PMB, Sigma-Aldrich) or polymyxin B nonapeptide (PMBN, Sigma-Aldrich) was dissolved in water to a concentration of 1 mg mL \(^{-1}\) and stored at −20 °C until further dilution to 100 μg mL \(^{-1}\) immediately before the AFM experiment was performed. Approximately 0.5 mL of water was carefully removed from the space between the AFM probe holder and glass substrate using the tip of a 1.0 mL standard micropipette (Fisher Scientific), and 0.5 mL of the peptide solution (100 μg mL \(^{-1}\)) was introduced to achieve the desired concentration of 50 μg mL \(^{-1}\) for the liquid environment surrounding the AFM probe. This concentration was chosen to be the value recommended by Sigma-Aldrich for clinical use of PMB, which is a factor of 25 higher than the MIC value for PMB,\(^{44}\) so that a definitive response could be observed, with all of the cells mechanically intact before exposure, and all of the cells mechanically compromised after exposure. Measurements were also performed using a much lower concentration of PMB (5.0 μg mL \(^{-1}\)). The peptide solution was added carefully to ensure that the same bacterium that was measured before the peptide treatment could be measured after the peptide treatment. Using this procedure, we observed only a small lateral drift in the AFM image (1–2 μm) after adding the peptide solution so that the same bacterial cell was easily identified.

**Optical microscopy measurements**

*Pseudomonas aeruginosa* PA01 bacteria were immobilized on poly-L-lysine-coated number 1 glass cover slides (thickness of 0.15 mm) following the same preparation procedure as for the AFM measurements. The glass slide was placed in a customized flow cell with temperature control, and bright-field microscopy images were collected using a 60 × objective on an Olympus IX71 inverted optical microscope. Prior to adding the antimicrobial peptides, the flow cell was filled with LB growth medium and heated to a temperature of 37 °C, providing conditions that are favorable for bacterial growth and division, and cells were observed to move, grow and divide. This observation indicates that the cells remain viable following our cell preparation procedure that is common to both the AFM and optical microscopy measurements. A small volume (~100 μL) of either PMB or PMBN dissolved in water to a concentration of 1 mg mL \(^{-1}\) was then added to the flow cell using a syringe to achieve the desired concentration of 50 μg mL \(^{-1}\) of the peptide, which was the same concentration as that used in the AFM experiment. After exposure to either PMB or PMBN for 30 min and at subsequent 30 min intervals, the fluid in the cell was exchanged with fresh LB growth medium using a syringe pump (KD Scientific Pump 100) using a flow rate of 1 mL min \(^{-1}\). For each experiment, a time series of the optical images was constructed using ImageJ. For cells exposed to PMB, no growth or motion of the cells was observed (see ESI Movie S1†), whereas for cells exposed to PMBN, the cells were observed to move, grow and divide (see ESI Movie S2†). These observations verified that exposure to PMB renders the bacterial cells non-viable, whereas the cells remain viable after exposure to PMBN.

**Results**

**AFM imaging of bacterial cells**

Atomic force microscopy (AFM) images of *P. aeruginosa* PA01 wild-type cells were collected before and after exposure to PMB or PMBN. Representative AFM deflection images, collected using a sharp AFM tip (radius of curvature \(R < 10 \text{ nm}\)), are shown in Fig. 1, and the corresponding AFM topography images and representative line scans are shown in ESI Fig. S1†. The surfaces of the untreated PA01 cells in Fig. 1(a) and (c) are relatively smooth (a root-mean-square (RMS) roughness of 1.3 nm averaged over a 100 nm × 100 nm area of the surface at the center of the bacterial cell, as measured on the topography images). After exposure to either 50 μg mL \(^{-1}\) PMB or 50 μg mL \(^{-1}\) PMBN, the cell surface roughness increased significantly (an RMS roughness of 8.1 nm for the upper cell in Fig. 1(b) that was collected 11 min after exposure to PMB, and an RMS roughness of 5.0 nm for the cell in Fig. 1(d) that was collected 16 min after exposure to PMBN). This increase in the cell surface roughness occurs quickly after exposure to the peptides, and could be due to the release of LPS molecules from the outer membrane as well as molecules from within the periplasmic (both PMB and PMBN) and cytoplasmic space (PMB)\(^{44}\) that can produce a decrease in the turgor pressure of the cell. Observation of roughening of bacterial cell surfaces after exposure to
antimicrobial compounds has been observed previously using AFM imaging, e.g. ref. 46 and 47.

Accompanying the increase in surface roughness, there is a small but measurable decrease in the height of the PMB-treated cells relative to their heights measured in water after long times of exposure to PMB, as shown in Fig. 1(e). To track changes in the height of the bacterial cells, we imaged the cells using a colloidal AFM probe with a large tip diameter of either 600 nm or 1 μm. This choice of tip allowed us to measure both the height and the viscoelastic properties of the cells as a function of the exposure time to the antimicrobial peptides. To characterize the changes in cell height with time, we fit the data to an exponential function for which we obtained a best-fit value of the characteristic time constant \( \tau \).

Histograms of cell heights measured before and after more than 1 h of exposure to PMB are shown in Fig. 1(f). In both cases, the distribution of cell heights is well defined. Although there is a small fraction of bacterial cells with significantly smaller cell heights (cell heights less than the average value by more than 20%) after 1 h of exposure to PMB, there is only a small decrease of 21 nm in the average cell height for the whole population in Fig. 1(f). We observed that the average cell height decreased more significantly by 104 nm after exposure to 50 μg mL\(^{-1}\) PMBN, with a significantly broader distribution of cell heights (ESI Fig. S1(c)†).

AFM measurements of the mechanical properties of bacterial cells

In the AFM-based creep deformation experiment, the force is applied rapidly to the bacterial cell, which allows the measurement of its elastic stiffness (force–displacement measurement). The applied force \( F_0 \) is then held constant and the displacement \( Z(t) \) of the AFM tip is measured, which allows the measurement of the viscoelastic properties of the cell (creep deformation measurement).

The effective spring constant \( k_1 \) of the bacterial cells was obtained from the slope \( k \) of the linear portion of the force–displacement curve during rapid loading (1.98 μm s\(^{-1}\)) of the bacterial cell. The value of \( k_1 \) was obtained by considering \( k \) to be the equivalent spring constant of the AFM cantilever (spring constant \( k_0 \)) in series with the bacterial cell (spring constant \( k_1 \), such that \( k_1 = k \times k_0/(k_0 - k) \)).

A typical creep deformation curve obtained for a P. aeruginosa PAO1 bacterial cell in water before exposure to PMB or PMBN is shown in Fig. 2(a). With \( F_0 \) held constant, the displacement of the AFM tip increases monotonically with time, asymptotically approaching a constant value within several seconds. The mechanical response of the cell to \( F_0 \) can be described by a very simple arrangement of springs and dashpots (inset to Fig. 2(a)) known as the standard solid model (SSM).37–40

For the SSM, the displacement \( Z(t) \) is given by:

\[
Z(t) = \frac{F_0}{k_1} + \frac{F_0}{k_2} \left[ 1 - \exp\left(-\frac{t}{\eta_2}\right) \right] \tag{1}
\]

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**Fig. 1** AFM deflection images of P. aeruginosa PAO1 bacterial cells (a) before and (b) after 11.4 min of exposure to 50 μg mL\(^{-1}\) PMB, and (c) before and (d) after 16 min of exposure to 50 μg mL\(^{-1}\) PMBN. The arrows indicate the same cells in parts (a) and (b), and in parts (c) and (d). (e) Cell height \( h \) versus time after exposure to PMB for one representative cell, showing a fast decrease in cell height over a time of several min; (f) histograms of cell heights before (light purple, \( h = 480 \pm 45 \) nm) and more than 1 h after (grey, \( h = 460 \pm 50 \) nm) exposure to 50 μg mL\(^{-1}\) PMB. The overlap between the two histograms is indicated by the darker colour.

**Fig. 2** (a) A representative creep deformation curve measured for a P. aeruginosa PAO1 cell in water, with a schematic diagram of the standard solid model shown in the inset. (b) A representative creep deformation curve measured for a P. aeruginosa PAO1 cell after 25 min of exposure to 50 μg mL\(^{-1}\) PMB, with a schematic diagram of the Burgers model shown in the inset. The black curves in (a) and (b) were calculated using the best fit parameters obtained from a least squares fit of the data to (a) the second term on the right hand side of eqn (1) (two parameter fit) and (b) the second and third terms on the right hand side of eqn (2) (three parameter fit).
where \( k_1, k_2 \) and \( \eta_2 \) correspond to the elements shown in the inset to Fig. 2(a). The best-fit values of \( k_1 \) were obtained from the force–indentation curves, and the best-fit values of \( k_2 \) and \( \eta_2 \) were obtained by fitting the creep deformation curves to the second term of the right hand side of eqn (1). The ratio \( \eta_2/k_2 \) defines a characteristic response time \( \tau \) for the delayed elastic response.

After exposure to either PMB or PMBN, the shape of the creep deformation curve undergoes an abrupt, qualitative change: instead of asymptotically approaching a constant value, the deformation continues to increase linearly with time (Fig. 2(b)). To describe this behaviour with a mechanical model, it is necessary to add another dashpot in series with the SSM (inset to Fig. 2(b)), known as the Burgers model. The displacement \( Z(t) \) is given by:

\[
Z(t) = \frac{F_0}{k_1} + \frac{F_0}{k_2} \left[ 1 - \exp \left( -\frac{t k_1}{\eta_2} \right) \right] + \frac{F_0}{\eta_1} t
\]

which has the same form as eqn (1) with an additional term on the right hand side that is determined by the viscosity \( \eta_1 \) and describes a contribution to \( Z(t) \) that is linear in time.

The linear increase in \( Z \) with time that is observed for times \( t > \tau \) after exposure to PMB and PMBN occurs because the AFM tip continues to move into the cell with time. This is a definitive signature for the loss of integrity of the cell envelope due to the action of PMB and PMBN. We can characterize the permeability of the cell envelope with the reciprocal of the viscosity \( \eta_1 \). Before exposure, the membrane permeability parameter \( 1/\eta_1 \) is essentially zero since the cell envelope is intact, and it increases following exposure, indicating that the cell envelope integrity has been compromised.

The loss of membrane integrity complicates the creep deformation experiment since pressing on the cell can release molecules from within the cell. This can be seen as a decrease in cell height for large applied forces in ESI Fig. S2(b) and (c).† As a result, the properties of the cell could change during the course of an experiment. To limit the number of molecules released due to pressing on the cell, we used forces that were as small as possible (<4 nN) to obtain reproducible measurements of the viscoelastic properties.

After exposure to PMB and PMBN, we determined the best fit value of \( k_1 \) from the force–displacement curve, and then fit the creep deformation data to eqn (2) to obtain the best-fit values of the delayed displacement \( \delta Z = F_0/k_2 \), the characteristic response time \( \tau = \eta_2/k_2 \) and the slope \( \alpha = F_0/\eta_1 \). Because the delayed elastic contribution to the total creep deformation described by eqn (2) occurs with a characteristic time \( \tau = \eta_2/k_2 \) that is much less than the total time for which the creep deformation is measured, it is possible to determine the \( \tau \) and \( \alpha \) values independently from the fitting procedure. The viscoelastic properties of the bacterial cell were obtained as follows: \( k_1 \) (measured from force–displacement curves), \( k_2 = F_0/\delta Z \), \( \eta_2 = k_2 \tau \), and \( 1/\eta_1 = \alpha/F_0 \).

**Long-term effects of exposure to cationic peptides**

To evaluate the long-term effects of exposure to the cationic peptides, we measured force–displacement and creep deformation curves on a large number of bacterial cells before and more than 1 h after exposure to either 50 \( \mu \)g mL\(^{-1} \) PMB or 50 \( \mu \)g mL\(^{-1} \) PMBN. The 1 h period was judged to be sufficient to observe long-term effects since the time of action of both PMB and PMBN was measured to be of the order of 10 min,† and this time of action is consistent with the time-resolved measurements presented below and in complementary AFM imaging.

![Fig. 3](image1.png) Histograms of viscoelastic parameters (a) \( k_1 \), (b) \( k_2 \), (c) \( \eta_2 \), and (d) \( 1/\eta_1 \) for *P. aeruginosa* PAO1 cells before and more than 1 h after exposure to 50 \( \mu \)g mL\(^{-1} \) of PMB. The viscoelastic parameters are defined in the schematic diagrams shown in Fig. 2. For each plot, the overlap between the two histograms is indicated by the darker colour.

![Fig. 4](image2.png) Histograms of viscoelastic parameters (a) \( k_1 \), (b) \( k_2 \), (c) \( \eta_2 \), and (d) \( 1/\eta_1 \) for *P. aeruginosa* PAO1 cells before and more than 1 h after being treated with 50 \( \mu \)g mL\(^{-1} \) of PMBN. The viscoelastic parameters are defined in the schematic diagrams shown in Fig. 2. For each plot, the overlap between the two histograms is indicated by the darker colour.
Following exposure to PMB, the centre of the distribution of the cells, and the distribution broadens significantly. Following exposure to PMBN, which was introduced at \( t = 0 \), the centre of the distribution shifted dramatically a short time (\( >5 \) min in Fig. 5). In contrast, after exposure to 50 \( \mu g \) mL\(^{-1} \) PMBN, the cells move and divide at rates that are indistinguishable from those measured before exposure (ESI Movie S2†).

Both before and after exposure to either PMB or PMBN, there are well-defined distributions for each of the viscoelastic parameters (Fig. 3 and 4, ESI Table S1†). The most striking result is the unambiguous signature of the loss of integrity of the bacterial cell envelope following exposure to either PMB or PMBN, which can be seen clearly in the histograms for the permeability parameter 1/\( \eta_1 \) (Fig. 3(d) and 4(d)). Before exposure, the distribution of 1/\( \eta_1 \) values is very narrow and centred about zero, corresponding to intact cell envelopes. For exposure to either PMB or PMBN, the 1/\( \eta_1 \) distribution shifts considerably to nonzero values, corresponding to permeable cell membranes, and is significantly broadened. Significantly, the shift in the distribution is considerably larger (by \( \sim60\% \)) for PMB than for PMBN.

Before exposure, the peaks in the \( k_2 \) histograms, obtained from the force–displacement measurements, are narrow. Following exposure to PMB, the centre of the distribution shifts to a slightly larger value (by \( \sim13\% \)), corresponding to stiffening of the cells, and the distribution broadens significantly. Following exposure to PMBN, the centre of the distribution shifts to slightly smaller values (by \( \sim10\% \)), corresponding to softening of the cells, and the distribution also broadens significantly.

The viability of the cells was drastically affected by exposure to 50 \( \mu g \) mL\(^{-1} \) PMB: optical microscopy measurements of bacterial growth and motility revealed that almost all cells were immobilized and unable to divide (ESI Movie S1†). In contrast, after exposure to 50 \( \mu g \) mL\(^{-1} \) PMBN, the cells move and divide at rates that are indistinguishable from those measured before exposure (ESI Movie S2†).

For exposure to either PMB or PMBN, the 1/\( \eta_2 \) distributions are quite broad before exposure to the cationic peptides, and shift to much smaller values following exposure to both PMB and PMBN. The relative widths of the distributions are approximately the same before and after exposure for both cationic peptides (ESI Table S1†).

**Time-resolved response of bacterial cells to cationic peptides**

To investigate the time scale of action of the cationic peptides, we collected force–displacement and creep deformation curves repeatedly after introducing the cationic peptides, providing a time resolution of 10 s. This enabled us to observe abrupt changes to the viscoelastic parameters, as can be seen in the plots of the time dependence of the viscoelastic parameters in Fig. 5 (exposure to 50 \( \mu g \) mL\(^{-1} \) PMB) and Fig. 6 (exposure to 50 \( \mu g \) mL\(^{-1} \) PMBN) for representative cells.

For exposure to PMB, abrupt changes occurred in all of the viscoelastic parameters after a short time (\( \sim5 \) min in Fig. 5). In particular, a dramatic, abrupt increase can be seen in 1/\( \eta_1 \) in Fig. 5(c), corresponding to the compromising of the integrity of the bacterial cell envelope. Therefore, the time-resolved creep deformation experiment allows the determination of the specific time at which the cationic peptide compromises the bacterial cell envelope. Large, abrupt decreases were also observed in \( k_1 \), \( k_2 \) and \( \eta_2 \) (Fig. 5(a) and (b)). Following abrupt

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**Fig. 5** Viscoelastic parameters for a representative P. aeruginosa PAO1 bacterial cell versus time of exposure \( t \) to 50 \( \mu g \) mL\(^{-1} \) PMB, which was introduced at \( t = 0 \). (a) instantaneous elasticity \( k_1 \) and delayed elasticity \( k_2 \) versus exposure time. The inset graphs are the corresponding creep deformation curves measured at the times indicated by the arrows numbered from 1 to 3, measured with a constant applied force of 3.0 nN; (b) delayed viscosity \( \eta_2 \) versus exposure time; (c) permeability parameter 1/\( \eta_1 \) versus exposure time.

**Fig. 6** Viscoelastic parameters for a representative P. aeruginosa PAO1 bacterial cell versus time of exposure \( t \) to 50 \( \mu g \) mL\(^{-1} \) PMBN, which was introduced at \( t = 0 \). (a) instantaneous elasticity \( k_1 \) and delayed elasticity \( k_2 \) versus exposure time. The inset graphs are the corresponding creep deformation curves measured at the times indicated by the arrows, measured with a constant applied force of 3.0 nN and with the interval between tick marks representing 10 nm along the \( y \)-axis and 2 s along the \( x \)-axis; (b) delayed viscosity \( \eta_2 \) versus exposure time; (c) permeability parameter 1/\( \eta_1 \) versus exposure time. The black dotted line is meant to guide the eye.
In retracting the AFM tip from bacterial cells exposed to either retraction studies of bacterial growth and motility (ESI Movie S2) cells remain viable, as shown in long-term optical microscopy integrity of the cell envelope following exposure to PMBN, the compared to that of PMB. Despite the small but nonzero loss of changes in each of the viscoelastic parameters, there was a gradual, partial recovery of $k_1$ and $1/\eta_1$ with time.

For exposure to PMBN, changes in the viscoelastic properties also occur rather abruptly, but the changes are smaller and take place after a significantly longer time of exposure. These results are consistent with the reduced antimicrobial efficacy of PMBN compared to that of PMB. Despite the small but nonzero loss of integrity of the cell envelope following exposure to PMBN, the cells remain viable, as shown in long-term optical microscopy studies of bacterial growth and motility (ESI Movie S2†).

**Force plateaus in force-separation curves during AFM tip retraction**

In retracting the AFM tip from bacterial cells exposed to either 50 $\mu$g mL$^{-1}$ PMB or PMBN, we measured long plateaus in the force-separation curves (Fig. 7(a)). In contrast, force plateaus were not observed for retraction of the AFM tip from cells before exposure to the peptides. For exposed cells, we defined two quantities, force step and breakage distance (Fig. 7(b)). The distribution of force steps (Fig. 7(c)) measured on a single cell had peaks at $\sim$80 pN and $\sim$160 pN; measurements on 20 different cells showed a similar distribution of force steps (inset to Fig. 7(c)). A fit of the measured breakage distances between 30 nm and 500 nm (Fig. 7(d)) to a single exponential gave a best-fit decay length of 140 nm.

**Discussion**

In the present study, we have shown that creep deformation measurements of bacterial cells can provide a direct measure of the loss of mechanical integrity of the bacterial cell envelope. The permeability parameter $1/\eta_1$ reveals the transition from an intact cell envelope, corresponding to $1/\eta_1 = 0$, to an envelope that is mechanically compromised or “leaky”, corresponding to $1/\eta_1 > 0$. Significantly, the increase in $1/\eta_1$ is approximately twice as large for exposure to PMB as it is for exposure to PMBN (Fig. 3(d) and 4(d)). This is consistent with PMBN disrupting only the OM, and PMB disrupting both the OM and CM, as inferred from protein release studies. This result suggests that the creep deformation experiment can provide quantitative information that can be used to discern differences between the mechanisms of action of different antimicrobial compounds.

The results of the time-resolved creep deformation experiments (Fig. 5 and 6) show that the increase in $1/\eta_1$ corresponding to the compromising of the bacterial cell envelope occurs abruptly, particularly for exposure to PMB. Abrupt changes also occur in all of the other viscoelastic parameters at the same time. Both PMB and PMBN bind to the lipid A head group of the LPS molecules, disrupting their ordering and subsequently removing them from the OM. It is likely that the cells can withstand the removal of some amount of LPS, replacing the molecules through synthesis and transport, but continued action and accumulation of the cationic peptides on the cell surface will eventually result in the cell being unable to maintain the integrity of the OM. Based on the abrupt changes observed in the experiments (Fig. 5 and 6), this occurs in a catastrophic manner. The abrupt nature of the changes to the mechanical properties may indicate that the peptides need to accumulate to a critical surface concentration, corresponding to a sufficiently high peptide/lipid ratio, before the abrupt changes are observed. Such a critical concentration was also inferred by Fantner et al., using fast AFM imaging on *E. coli* cells, in which the cell surface remained smooth until an abrupt increase in roughness was observed at a specific time (between 1 and 6 min) following exposure to the CM15 peptide. The gradual decrease in cell stiffness $k_1$ with time observed for exposure to PMBN (Fig. 6) may also indicate the gradual accumulation of PMBN on the OM before the rapid change in the viscoelastic parameters at an exposure time of $\sim$20 min. Computer simulations of the interaction of cationic antimicrobial peptides with cell membranes have also indicated that peptides accumulate gradually on the membrane before an abrupt insertion into the membrane.

The changes in the viscoelastic parameters occur more quickly for exposure to PMB than for PMBN, which is consistent with the promoted uptake mechanism proposed for the translocation of PMB molecules across the OM. PMBN lacks the ability to cross the OM, and this produces smaller changes in the mechanical properties of the cell that occur after longer times of exposure.
We can interpret the results of the present study by attributing the parameters of the viscoelastic models shown in Fig. 2 to different components of the bacterial cells. One might expect that permeabilization of the cytoplasmic membrane due to the action of PMB would result in a decrease in the turgor pressure and a correspondingly smaller value of the cell stiffness \( k_2 \), where \( k_2 \) is the shift of the \( k_2 \) distribution following 1 h of exposure to PMB shown in Fig. 3(a) for the recovery of the \( k_2 \) values after exposure of the wild type strain. This probably leads to some entry of water into the periplasm that, as for the wild type cells exposed to PMB or PMBN, results in reduced values of \( k_2 \) and \( \eta_2 \). This result is consistent with the delayed elastic response for \( P. aeruginosa \) cells being dominated by the gel-like properties of the periplasm.

AFM creep deformation measurements performed at a significantly lower PMB concentration of 5.0 \( \mu \)g mL\(^{-1} \) (ESI Fig. S6†) demonstrated the sensitivity of the experiment, and revealed important differences between the low and high PMB concentration results, most notably a significantly larger decrease in \( k_2 \) and significantly smaller increase in \( 1/\eta_2 \). These differences are likely due to a different mechanism of action of PMB at low concentrations for which the cytoplasmic membrane is not depolarized.\(^{50,51} \)

We can also comment on the origin of the other viscoelastic parameters in the models shown in Fig. 2. The delayed elastic response is due to the more liquid-like components of the cell envelope, such as the hair-like O-antigen side chains of LPS molecules on the OM or the gel-like periplasm between the OM and CM. If the O-antigen side chains play an important role in the delayed elastic response, one might expect that cells with LPS molecules that lack O-antigen side chains would have a significantly larger \( k_2 \) value (corresponding to a smaller creep deformation in eqn (1)) than cells that have the O-antigen side chains. Previously, we have performed AFM creep deformation experiments on several \( E. coli \) strains, including a K12 strain,\(^{19} \) which lacks O-antigen side chains, and a strain\(^{38} \) that has O-antigen side chains. In these measurements, the \( k_2 \) values were the same to within experimental uncertainty and the \( \eta_2 \) value was increased by only a factor of 1.6 in the presence of the O-antigen side chains. Since the differences were not very large, it is likely that the contribution of the O-antigen side chains to the delayed elastic response is not very significant.

To further investigate the LPS contribution to the delayed elastic response in \( P. aeruginosa \) cells, we performed AFM creep deformation experiments on \( P. aeruginosa \) PA01 \( wapR \) LPS mutant cells, which lack the LPS O-antigen side chains.\(^{54,55} \) As discussed above, if the delayed elastic response observed for wild type cells was due to the LPS O-antigen side chains, we would expect that the \( wapR \) LPS mutant cells would have a larger value of \( k_2 \). However, we measured \( k_2 \) and \( \eta_2 \) values for the \( wapR \) LPS mutant cells (ESI Fig. S5†) that were significantly less (by roughly a factor of 2) than those values measured for the wild type cells (see ESI Table S1†). These measurements are complicated somewhat by the small, nonzero permeability of the \( wapR \) cells, which is perhaps due to their reduced ability to withstand a pure water environment relative to that of the PA01 wild type strain. This probably leads to some entry of water into the periplasm that, as for the wild type cells exposed to PMB or PMBN, results in reduced values of \( k_2 \) and \( \eta_2 \). This result is consistent with the delayed elastic response for \( P. aeruginosa \) cells being dominated by the gel-like properties of the periplasm.

Very large reductions in both \( k_2 \) and \( \eta_2 \) were produced by exposure of the wild type \( P. aeruginosa \) PA01 cells to either PMB or PMBN (reduction by factors between 3.3 and 5.9; see ESI Table S1†, Figs. 3 and 4). These large changes can be understood in terms of the permeabilization of the OM by the peptides that causes the release of periplasmic proteins.\(^{24,53} \) The release of proteins is likely accompanied by the dilution of the periplasm by water entering through defects formed in the OM, which should decrease both \( k_2 \) and \( \eta_2 \) and make the mechanical response of the periplasm more water-like.

We find that the measured values of the delayed elastic parameters and the permeability parameter are negatively correlated (ESI Fig. S4†). This negative correlation implies that larger values of the permeability parameter correspond to a more water-like periplasm with smaller values of \( k_2 \) and \( \eta_2 \), which is consistent with the action of the antimicrobial peptides as permeabilizers of the OM.

Our observation of long force plateaus during retraction of the AFM tip from cells exposed to PMB (Fig. 7(a)) and PMBN is another indication that the OM of the cells has been disrupted. This observation is consistent either with the formation of lipid nanotube tethers\(^{56–59} \) between the AFM tip and the cell surface, with rupture occurring when the lipid reservoir was exhausted, or with the pulling of individual macromolecules from the OM or periplasm. Since we observe a significant number of breakage distances that are considerably longer than the contour length of the proteins (up to 500 nm for the measured breakage distances in Fig. 7(d) versus \(<100 \text{ nm} \) for the contour length of typical outer membrane and periplasmic proteins), the results presented in Fig. 7 are most likely due to the formation of lipid tethers but could also be due to the pulling on oligomers of proteins.\(^{60} \) In either case, the observation of force plateaus in the retraction curves only after exposure to either PMB or PMBN indicates that the peptides caused sufficient disruption of the OM through removal of LPS molecules. If we interpret the force plateaus in terms of the formation of lipid tethers, the main 80 pN peak in the force step histogram
(Fig. 7(c)) would correspond to a lipid tether of radius 6.4 nm, and the characteristic decay length of the breakage distance distribution (140 nm from Fig. 7(d)) would correspond to a tether lifetime of 70 ms.

In the present study, the bacteria were suspended in water to improve the electrostatic adhesion of the negatively charged cell surfaces to the positively charged layer of poly-l-lysine or MAP. Pseudomonas aeruginosa PAO1 cells are able to withstand the osmotic stress introduced by suspension in water, as we have shown previously. In the present study, we also have direct indications of the mechanical integrity of the Pseudomonas aeruginosa PAO1 cells suspended in water before exposure to the antimicrobial peptides: (1) the plateau in the deformation observed in the creep deformation curves (Fig. 2(a)); (2) the lack of force plateaus observed during retraction of the AFM tip (Fig. 7, top curve); and (3) from the optical microscopy studies before and after exposure to the peptides. In preparing the samples for the optical microscopy studies, we were careful to subject the cells to the same preparation procedure as for the AFM measurements (centrifugation, re-suspension in Milli-Q water) before exposing them to the growth medium. Because the cells that are then re-suspended in the growth medium can move, grow and divide following the cell preparation procedure, this demonstrates that the bacterial cells withstand the cell preparation procedure that is common to both the AFM and optical microscopy measurements.

We have interpreted the results of the present study in terms of a viscoelastic response to a constant applied force. Recently, the theory of poroelasticity has been applied to the study of the mechanical properties of eukaryotic cells. We have considered this possible interpretation of the present results, but we find that they cannot be explained in terms of the theory of poroelasticity, as discussed in the ESL. In brief, because the indentations are so small, the times associated with the poroelastic response are very small compared with the characteristic response times measured in the creep deformation experiment. In addition, a force–relaxation experiment reveals a simple single exponential relaxation for the bacterial cells. Taken together, these results indicate that the viscoelastic models described above are appropriate for the interpretation of our data.

Summary and conclusions

We have presented a detailed study of the effect of two structurally related cationic peptides on the viscoelastic properties of P. aeruginosa PAO1 cells. AFM creep deformation experiments provide a distinctive signature of the loss of mechanical integrity of the bacterial cell envelope following the introduction of the peptides. By measuring changes in the viscoelastic properties before and after 1 h of exposure to the peptides, as well as time-resolved measurements, we obtained detailed information on four different viscoelastic parameters that we attribute to different components in the bacterial cell. Quantitative comparison of the permeability of the cells after 1 h of exposure to PMB and PMBN reveals that the magnitude of the change in permeability can be related to the action of the peptides on the outer and cytoplasmic membranes. In addition, we observed differences between the effects of exposure to high and low concentrations of PMB that can be attributed to the depolarization of the cytoplasmic membrane, i.e. a different mechanism of action, at the high concentration. By using specific peptides and bacteria, we have illustrated that our technique yields quantitative information concerning the known mechanism of action of the peptides. We note that this technique is versatile and holds great promise for providing unique, crucial information for identifying the mechanism of action of novel antimicrobials.

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Notes and references

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