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RESEARCH NOTE

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Real-time multimodal imaging of daptomycin action on the cell wall of adherent *Staphylococcus aureus*

Alexis Canette^{1*}, Rym Boudjemaa², Julien Deschamps³, Karine Steenkeste², Christian Marlière², Romain Briandet^{3*} and Marie-Pierre Fontaine-Aupart²

Abstract

Objectives This study investigated the efficacy of daptomycin against adherent *Staphylococcus aureus* (*S. aureus*), a common colonizer of medical devices that leads to severe infections. For the first time, we evaluated the bactericidal effects of daptomycin on *S. aureus* immediately after adhesion, mimicking early-stage contamination of biomaterials. Time-kill curve assay and confocal laser scanning microscopy (CLSM) were used to analyze the process dynamics. In addition, atomic force microscopy (AFM) and scanning electron microscopy (SEM) were employed to elucidate daptomycin-induced structural changes in the bacterial cell wall.

Results description Daptomycin, at clinically relevant concentrations, rapidly eradicated adherent bacteria in the exponential growth phase, demonstrating an efficiency comparable to its action against planktonic cells. Prolonged exposure to the antibiotic caused marked alterations in the bacterial cell wall, including surface roughening and perforation, as revealed by multimodal imaging. However, daptomycin effectiveness diminished as biofilm formation progressed, underscoring the need for further exploration of optimized clinical strategies.

Keywords Daptomycin, *Staphylococcus aureus*, Atomic force microscopy, Scanning electron microscopy, Confocal laser scanning microscopy, Bacterial cell wall, Biofilm, Antibiotic tolerance

Introduction

Bacterial colonization of biomaterials poses a significant challenge in clinical settings, often leading to persistent and severe infections associated with medical devices [1, 2]. These infections, particularly those caused by *S. aureus*, have a substantial impact on patient outcomes, including prolonged hospital stays, increased morbidity, and mortality [3]. The economic burden is also significant, with increased healthcare costs due to the necessity for extended treatments and additional interventions [3, 4]. For example, *S. aureus* is a leading cause of prosthetic vascular-graft infections, which have been associated with heightened complications and healthcare expenses [4].

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Despite efforts to prevent bacterial adhesion and biofilm formation, it remains challenging [5]. Antibiotics are widely used to mitigate this issue, but their effectiveness is often compromised by the rise of antibiotic-resistant pathogens that persist even in medicated environments [1, 6]

This study draws on our previous work [7] to address a critical gap in understanding of daptomycin activity against *S. aureus* during the initial stages of bacterial attachment to surfaces. Daptomycin, a lipopeptide antibiotic, has emerged as a critical therapeutic option for treating multidrug-resistant *S. aureus* [8]. Daptomycin efficacy relies on its ability to oligomerize within membranes, forming pores that subsequently lead to cell death. Such a process depends on the membrane fatty acids composition, explaining part of the success of daptomycin treatment on bacteria in their exponential growth phase and its failure on bacteria in the stationary phase or included in mature biofilms [9]. To our knowledge, no data was reported on daptomycin efficiency against adhered bacteria on a surface, which is essential from the perspective of antibiotic use against surface contamination. The combination of time-kill curve assays, live/dead assays by confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) enabled us to examine both the functional and structural responses of *S. aureus* at the nanoscale level, offering a comprehensive analysis of daptomycin activity during early bacterial attachment.

Main text

Strain and growth conditions

S. aureus ATCC 27217 was stored in aliquots at -20°C and subsequently subcultured in Trypticase Soy Broth (TSB; BioMérieux, France) at 37°C . Cells reached the stationary phase after an initial 8 h of culture followed by an overnight incubation. Exponentially growing cells were produced by a subsequent 3-hour subculture in TSB at 100 rpm. The cultures were then centrifuged twice, washed with sterile 9 g/L NaCl solution, and resuspended in TSB.

Daptomycin efficacy testing

The antibacterial activity of daptomycin was evaluated on *S. aureus* in different growth states: exponential, stationary, and within 24-hour biofilms [10]. This study also extended to exponentially growing cells adhered to surfaces. To quantify adhered bacteria, 200 μL of the bacterial suspension (initial concentration $\sim 10^6$ CFU/mL, as verified by an optical density reading of 0.05 at 600 nm) was added to each well of a polystyrene 96-well microtiter plate with a μClear base (Greiner Bio-One, France) designed for high-resolution fluorescence imaging. The

plate was incubated at 37°C for 90 min to allow bacterial adhesion to the well bottoms. After adhesion, nonadherent cells were removed by rinsing with 9 g/L NaCl, and wells were refilled with TSB supplemented with 50 mg/L calcium required to facilitate daptomycin action [9] and corresponding to blood calcium concentration [11]. Daptomycin was introduced at a clinically relevant concentration of 20 $\mu\text{g}/\text{mL}$ [12].

Time-kill curve assay was quantified at baseline (T0) and at 3, 6- and 24-hours post-treatment using the colony-forming unit (CFU) method. Adhered cells or biofilms were scraped off, resuspended and homogenized in a sterile NaCl solution, and centrifuged twice. Serial tenfold dilutions were prepared and plated on Trypticase Soy Agar (TSA) plates to quantify viable culturable bacteria at each time point for planktonic bacteria [10]. The detection threshold of viable culturable cells was 100 CFU/mL.

Live/dead assays by CLSM

After bacterial adhesion for 90 min in the 96-well microtiter plates described above, the wells were rinsed to remove nonadherent cells and then refilled with 200 μL of TSB supplemented with either (i) 20 μM propidium iodide (PI), a red nucleic acid dye that can easily penetrate cells with compromised plasma membranes, for control experiments, or (ii) 20 μM PI, 20 $\mu\text{g}/\text{mL}$ daptomycin and 50 $\mu\text{g}/\text{mL}$ CaCl_2 to evaluate the effect of daptomycin on the adhered cells. The prepared microplate was immediately transferred to a confocal laser scanning microscope (Leica Microsystems SP8, Germany) equipped with a water immersion 63x NA 1.2 objective lens at the INRAE MIMA2 platform (<https://doi.org/10.15454/1.5572348210007727E12>) for time-lapse imaging. Images stacks (512 \times 512 pixels, 184.5 \times 184.5 μm , 1 μm z-step) were captured every 30 s for 20 min, using both transmitted light and the red fluorescence channel for PI detection. These images were collected from three wells to ensure reproducibility. Post-acquisition analysis was conducted using ImageJ software, enabling a quantitative evaluation of the bactericidal impact of daptomycin.

AFM experiments

The protocols for bacterial immobilization, AFM setup, data acquisition, and analysis methods were adapted as previously described [7]. Briefly, AFM studies were conducted using a Nanowizard III (JPK Instruments AG, Berlin, Germany) mounted on an inverted microscope (AxioObserver Z1, Carl Zeiss, Göttingen, Germany). AFM measurements were taken in fast-speed approach/retract mode (Quantitative Imaging[®] (QI) mode, JPK), allowing local mechanical property assessment (Young's moduli) through force spectroscopy. Bacterial suspensions, harvested in exponential or stationary growth

phases, were deposited on cleaned ITO surfaces and allowed to adhere for 1 h at 30 °C without prior centrifugation. Samples were then gently rinsed and covered with sterile aqueous NaCl (9 g/L) supplemented with 50 mg/L CaCl₂. Daptomycin was added at a concentration of 20 µg/mL directly onto the samples to minimize disturbance. AFM probing was performed before and after antibiotic application to the same cells to assess surface integrity and minimize variability between samples. Experiments were performed using two independent bacterial cultures.

SEM experiments

Planktonic *S. aureus* cultures in both the exponential growth and stationary phases were treated with daptomycin at a final concentration of 20 µg/mL. After 30 min or 3 h antibiotic exposure (no treatment for the control condition), the cells in TSB were fixed with 4% v/v glutaraldehyde in distilled water for 1 h, followed by centrifugation at 5000 rpm for 10 min. The fixed cells were then transferred to aluminium coupons and stored overnight at 4 °C. After fixation, samples were washed with 0.1 M sodium cacodylate buffer, then sequentially dehydrated in an ascending ethanol series, and dried using a critical point dryer (Leica CPD300). Dried samples were mounted on aluminium stubs with adhesive tabs and coated with a 6 nm layer of platinum using a Leica ACE600 sputter coater under argon plasma conditions.

Observations were conducted at the IBPS EM platform using a Zeiss FEG Gemini 500 SEM operated at 2 kV with a 20 µm aperture and high current setting. The imaging setup included a 50%-50% mix of secondary electrons with the lens and the chamber detectors, images with 1024 × 768 pixels, a pixel dwell time of 1.6 µs, and a line average of 10.

Results and discussion

Daptomycin bactericidal activity against adhered *S. Aureus*

Previous reports have indicated that the bactericidal activity of daptomycin varies with the growth state of *S. aureus* [10]. In planktonic cells in the exponential growth phase, daptomycin achieved a 2-log reduction in bacterial count within 6 h and reached a 4.2-log reduction at 24 h, demonstrating strong bactericidal activity (Fig. 1).

Conversely, only a bacteriostatic effect (inhibition of bacterial growth) was observed within the first 6 h for stationary-phase planktonic cells and cells within biofilms, with bacterial regrowth detected at 24 h (Fig. 1). This study further revealed that adhered exponentially growing cells exhibit similar sensitivity to daptomycin as planktonic cells within the first 6 h; however, these adhered cells show regrowth over 24 h, eventually returning to their initial concentrations (Fig. 1). These results align with the established mode of action of daptomycin, which targets actively dividing cells more effectively than the slow-growing bacteria characteristic of biofilms [10].

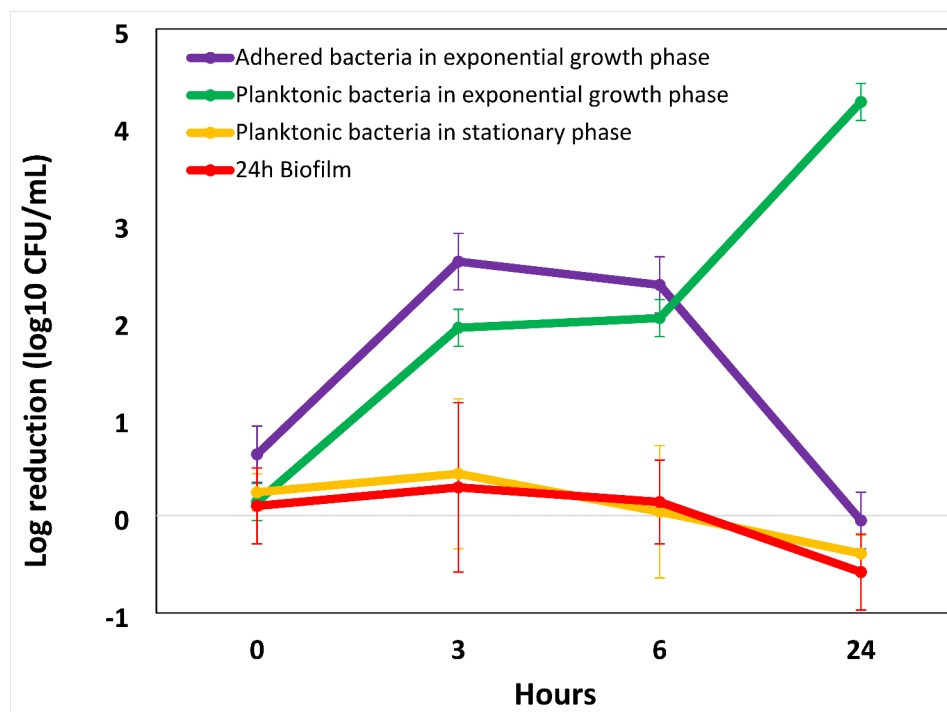


Fig. 1 Bactericidal activity of daptomycin, measured as bacterial log reduction, on adhered exponential growth *S. aureus* bacteria as a function of time by comparison to antibiotic efficiency against planktonic bacteria in their exponential growth and stationary phases and forming a biofilm as reported in [10]. Bars represent the standard deviations calculated from at least 4 independent experiments

After 6 h, as biofilm development progresses, the physiological state of the bacterial population shifts, reducing susceptibility to daptomycin.

Dynamics of daptomycin against adhered *S. Aureus*

The *S. aureus* bacteria that adhered to microplates were imaged and quantified by transmitted light, and the effect of daptomycin was assessed by the quantification of IP-labelled bacteria via CLSM. The corresponding real-time daptomycin activity, followed by CLSM measurements, is illustrated in Fig. 2A.

We observed that within 5 min after antibiotic addition, ~85% of the adhered exponentially growing cells were damaged (limit of observation by CLSM), highlighting the rapid daptomycin access on the cytoplasmic membrane.

It was hypothesized that these membrane-associated processes impaired cell envelope synthesis [13]. We then utilized the AFM method to investigate the effect of daptomycin on the cell wall remodelling of adhered exponentially growing *S. aureus* bacteria in real time. Before antibiotic exposure, we characterized the bacterial topographical and mechanical properties (Figure S1), confirming the presence of well-shaped exponential-growing bacteria with a herring-bone surface structure related to specific Young's moduli values following our previously published data [7]. Interestingly, we observed after the application of daptomycin that the antibiotic rapidly altered the nanoscopic herringbone pattern of the cell surface, which became rougher in less than

10 min (Fig. 2B): a significant increase in the height difference values was measured (~1 to ~10.2 ± 1.7 nm) and remained stable over 60 min (the maximum time of observation before more significant topographic alterations occurred and contaminated the AFM tip). During this time, the overall stiffness of the cell surface did not change (Figure S1), suggesting localized mechanical disruptions rather than uniform alterations across the cell envelope, as also highlighted by SEM analysis.

Bacterial cell wall structural response to daptomycin action

SEM provided complementary and interesting information on *S. aureus* cell wall morphology and degradation upon daptomycin treatment compared with the untreated control (Fig. 3A).

After the bacteria were exposed to daptomycin for 30 min, SEM image analysis revealed that only ~4% of the cells exhibited severe surface topography alterations (Fig. 3B), while ~85% of them were dead, as revealed by CLSM (see above). The cell surface modifications appeared as holes (Fig. 3B'), and some impressive cells burst at the septal level (Fig. 3B'). After 3 h of antibiotic treatment (Fig. 3C), the percentage of bacteria modified on their surface significantly increased (15%). The wall was completely removed from the septal area (Fig. 3C') for the most spectacular changes, and the coccus form was hard to recognise. The internal structure also seemed altered and leaked at the top of the bacteria. Another typical modification of the surface topography is the appearance of several perforations on the surface, as observed in

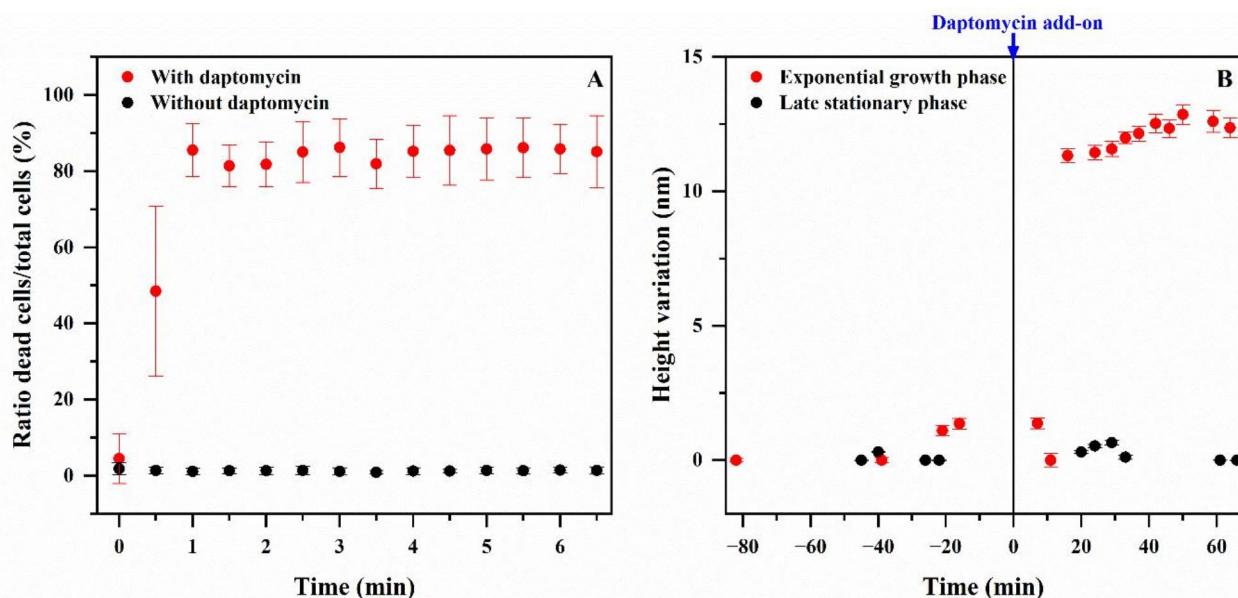


Fig. 2 (A) Confocal Laser Scanning Microscopy (CLSM) kinetics demonstrating the rapid impact of daptomycin on the cytoplasmic membrane of *S. aureus* cells. (B) Kinetics of bacterial height variation derived from Atomic Force Microscopy (AFM) measurements before and after daptomycin treatment on adhered *S. aureus* cells, emphasizing surface disturbances in the exponential growth phase by comparison to the late stationary phase

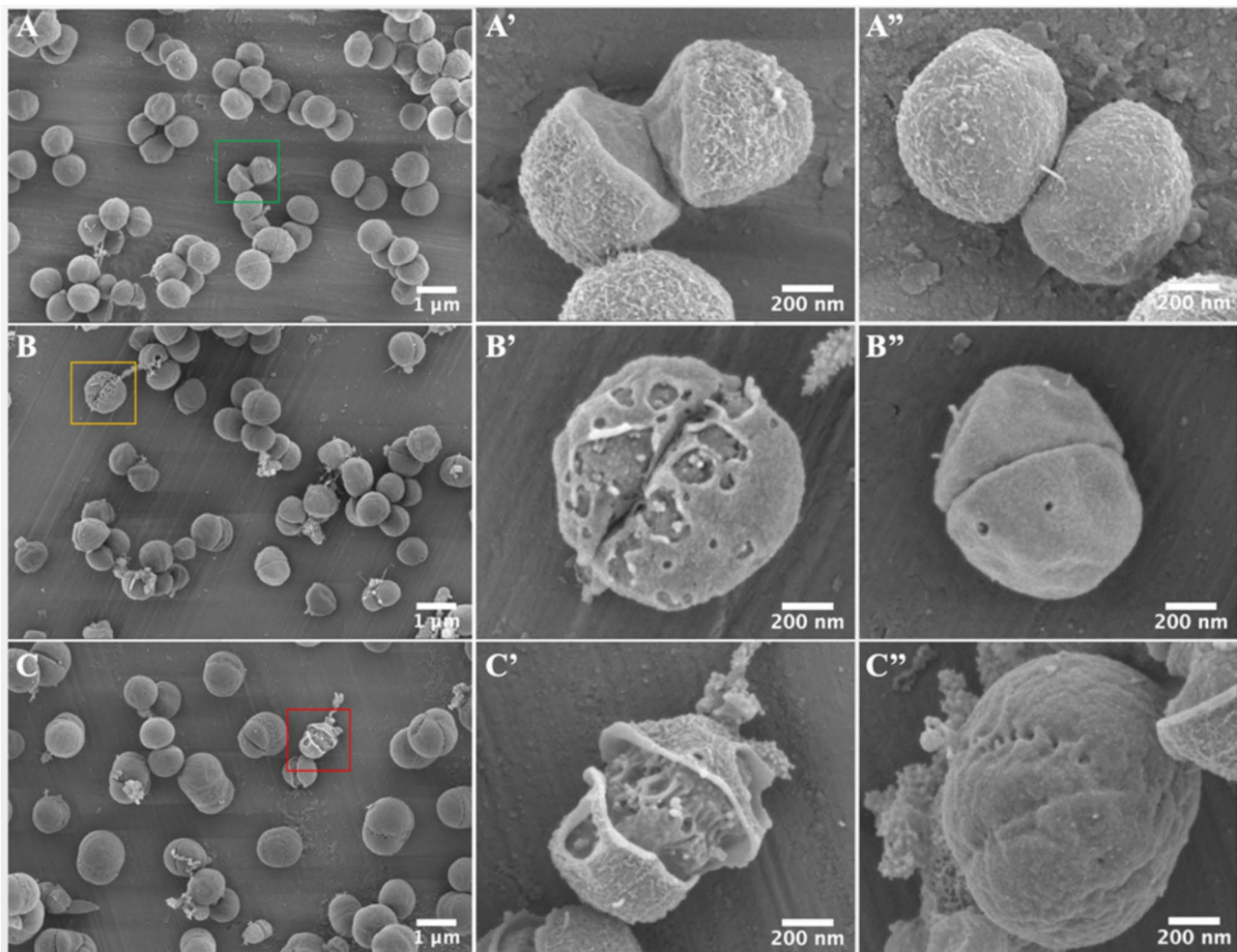


Fig. 3 Daptomycin-induced effects on the morphology of *S. aureus* cells in the exponential growth phase. **(A)** 10 000 X magnified field of bacteria without daptomycin (control condition). **(A')** 80 000 X magnified field focused on the bacterium in the green insert. **(A'')** Other 80 000 X magnified field focused on a bacterium of the control condition. **(B)** 10 000 X magnified field of the 30 min daptomycin exposition condition. **(B')** 80 000 X magnified field focused on the bacterium in the orange insert. **(B'')** Other 80 000 X magnified field focused on a bacterium of the 30 min daptomycin exposition condition. **(C)** 10 000 X magnified field of the 3 h daptomycin exposition condition. **(C')** 80 000 X magnified field focused on the bacterium in the red insert. **(C'')** Other 80 000 X magnified field focused on a bacterium of the 3 h daptomycin exposition condition. The magnification corresponds to the microscope field compared to the Polaroid 545 reference

Fig. 3C''). Nevertheless, most of the cells appeared to have intact surfaces, highlighting a slow antibiotic effect on the cell wall. Notably, the conventional SEM approach does not allow observation of the variation in the herringbone height highlighted by AFM.

In coccoid cells such as those of *S. aureus*, new peptidoglycan is inserted only at the dividing septum [14]. We speculate that exposure to daptomycin leads to an accumulation of peptidoglycan and teichoic acid precursors synthesized in the cytoplasm under conditions where the translocation of lipid-linked precursors from the cytoplasmic side to the outer side of the membrane is disrupted. These changes suggest a disruption in peptidoglycan and teichoic acid precursor translocation, possibly leading to mechanical rupture of the cell wall under pressure. The absence of any effect of the antibiotic on *S.*

aureus cell surface morphology in the stationary phase (Figure S2) supports this hypothesis (in accordance with [10]).

Conclusion

This study provides a novel insight into the efficacy of daptomycin against exponential stage *S. aureus* adhering cells. By utilizing a variety of nano- and microscopic imaging techniques, we tracked the kinetics of cellular processes following daptomycin exposure. Notably, our findings indicate that antibiotic efficacy declines after 6 h, likely due to emerging tolerance mechanisms as biofilm-promoting conditions develop. These results underscore the need for further research to design optimized clinical strategies to mitigate these limitations.

Limitations

This study was conducted under model conditions, limiting its direct application to clinical settings. To bridge this gap, future research should investigate the behaviour of clinical isolates with varying susceptibility profiles adhering to medical devices such as catheters, implants, prostheses, etc. Furthermore, our findings underscore the need to evaluate higher concentrations of daptomycin in antibiotic lock therapies designed for biomedical surfaces.

Abbreviations

FDA	Food and Drug Administration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
SEM	Scanning electron microscopy
AFM	Atomic Force Microscopy
CLSM	Confocal laser scanning microscopy
TSB	Trypticase Soy Broth
CFU	Colony Forming Units
PI	Propidium iodide

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13104-025-07130-5>.

Supplementary Material 1

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Author contributions

SEM experiments, conceptualization and methodology, Reviewing and editing.RyB: AFM and microbiology experiments, conceptualization and methodology.JD: CLSM experiments.KS: AFM experiments, Formal analysis and data curation, Figure preparation, Validation and supervision, Reviewing and editing.CM: AFM experiments, Reviewing and editing.RoB: conceptualization and methodology, Reviewing and editing.MPFA: writing the original draft preparation, validation and supervision, funding acquisition, project administration.All authors reviewed the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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