Correlative ex situ and Liquid-Cell TEM Observation of Bacterial Cell Membrane Damage Induced by Rough Surface Topology

Background: Nanoscale surface roughness has been suggested to have antibacterial and antifouling properties. Several existing models have attempted to explain the antibacterial mechanism of nanoscale rough surfaces without direct observation. Here, conventional and liquid-cell TEM are implemented to observe nanoscale bacteria/surface roughness interaction. The visualization of such interactions enables the inference of possible antibacterial mechanisms.

Methods and Results: Nanotextures are synthesized on biocompatible polymer microparticles (MPs) via plasma etching. Both conventional and liquid-phase transmission electron microscopy observations suggest that these MPs may cause cell lysis via bacterial binding to a single protrusion of the nanotexture. The bacterium/protrusion interaction locally compromises the cell wall, thus causing bacterial death. This study suggests that local mechanical damage and leakage of the cytosol kill the bacteria first, with subsequent degradation of the cell envelope.

Conclusion: Nanoscale surface roughness may act via a penetrative bactericidal mechanism. This insight suggests that future research may focus on optimizing bacterial binding to individual nanoscale projections in addition to stretching bacteria between nanopillars. Further, antibacterial nanotextures may find use in novel applications employing particles in addition to nanotextures on fibers or films.

Keywords: liquid TEM, graphene liquid cell, antibacterial nanopatterns, antibacterial surface topology, antibacterial microparticles

Introduction

Antibacterial treatments are frequently implemented in modern medicine, agriculture, and antimicrobial textiles.1,2 Bacteria are, however, capable of developing resistance to many antibacterial treatments.3,4 Conventional antibiotics typically act by preventing the upkeep of the cell wall, interfering with the bacterial metabolism, or by inhibiting the synthesis of nucleic acids or proteins.5 Antibiotics may also depolarize the cell membrane, allowing ion influx.6 This change in ion concentration can kill bacteria.7 Antibiotic resistance occurs through limiting uptake of the antibiotic, removal of the antibiotic from within the bacteria, inactivation of the antibiotic through chemical modification or hydrolysis, or modification of the antibiotic’s target.8,9 In gram-negative bacteria, the outer plasma membrane provides intrinsic protection against the uptake of many antibiotics.10 Antibiotics primarily depend on the presence of porin proteins in the plasma membrane.11 These porin proteins facilitate the transport of hydrophilic antibiotics across the cell envelope.11
Mutations in the porin proteins may prevent the transportation of antibiotics.11 Bacteria may also reduce the number of porins.11 In contrast, gram-positive bacteria lack the outer plasma membrane and are less likely to limit the uptake of antibiotics.12 Gram-positive bacteria thus primarily depend on removal of the antibiotic, destruction of the antibiotic, or inactivation of the antibiotic via chemical modification.5 Alternatively, bacteria may alter the target of the antibiotic to prevent the antibiotic effects.5

Three general approaches to battle antibiotic-resistant bacteria exist: 1) continued development of conventional antibiotics, to which bacteria continue to adapt, 2) sterilization techniques using chemical agents, such as bleach, other harsh chemicals, or heating and cleaning of surfaces; such approaches have limited medical applications, require continuous reapplication, and may have detrimental environmental ramifications. 3) Novel fundamentally-different antibacterial treatments that rely on silver or specific surface topologies.13–19 To this end, bacteria were shown to develop resistance to silver treatments.20,21 Silver may also present an ecological hazard similar to that posed by chemical or antibiotic treatments.22 Regardless of whether an antibacterial treatment features an antibiotic drug, strong chemical, or silver, introduction of substances toxic to bacteria may be inherently caustic and induce environmental damage.16,23

Antibacterial surface topologies were first observed in nature. Cicada wings and dragonfly wings exhibit nanotextures which induce bacterial death and reduce bacterial fouling.24–27 These antibacterial nanotextures may be synthesized on a variety of biodegradable, non-toxic, and non-pathogenic materials without the presence of any bactericidal chemical or elemental agent.28 In previous works, Serrano et al explored nanotexturing of biomedical sutures,28 which showed reduced bacterial attachment, suggesting the sutures would be less likely to transmit infections prior to implantation.28 However, nanostructured cicada wings were previously shown to kill bacteria with a physical mechanism rather than by preventing bacterial adhesion.26

The bactericidal property of nanopillar surface topologies has been proposed to occur through several mechanisms: Pogodin et al proposed that bacteria bind to the top of nanopillars.26 The bacteria then attempt to bind below the surface and are consequently stretched between the nanopillars.26 This stretching of the cell membrane supposedly causes rupture of the cell wall and induces bacterial death.26 In both gram-negative and gram-positive bacteria, the cell wall includes a layer of peptidoglycan, which provides mechanical support.29 In gram-positive bacteria, the cell envelope includes the outer cell wall, an intermediate periplasmic space, and an interior plasma membrane.29 In gram-negative bacteria, the cell envelope includes an outer lipopolysaccharide and protein layer, an intermediate periplasmic space, a middle cell wall, a second periplasmic space, and an interior plasma membrane.29 Linklater et al proposed another mechanism, in which the bacteria induce mechanical forces on the surface of nanopillars upon binding.30 These forces then deflect the nanopillars, which then induce strain on the bacteria upon relaxation of the pillars.30 This strain damages the cell wall and induces the observed cell death.30 While nanopillar surfaces were experimentally shown via traditional fluorescent light microscopy and scanning electron microscopy (SEM) to induce bacterial death, the mechanism of bactericidal activity was mathematically inferred in both Linklater et al and Pogodin et al rather than demonstrated experimentally.26,30 In another mechanism, Michalska et al proposed that black silicon nanopillars directly penetrate the cell wall of the bacterium.31 However, Linklater et al later suggested that the bacterial death observed in Michalska et al occurred via the strain mechanism.30,31

Additional studies have focused on mathematical modeling of bacteria/nanotexture interaction.32–34 By necessity, these mathematical models included assumptions such that the stiffness of the bacteria may be modeled as a single-layer of peptidoglycan, that deformation of the bacterial cell wall does not induce other metabolic processes (eg, apoptosis) through programmed cell death, and that the bacteria binding to the surface occurs through purely physics-based methods, such as hydrophobic interactions and Van der Waals forces. The initial bacteria/surface contact is indeed dependent on physical factors, such as surface charge and wettability (hydrophilicity/hydrophobicity).35–39 However, bacteria/surface binding occurs in a second stage, wherein adhesion forces far exceed the forces in the initial contact period and are dominated by multiple adhesion proteins and attachment pili.35–39 Bacterial binding of nanotextures is an active process and depends on the metabolic rate, which confirms the active role of protein adhesion in bacteria/nanotexture binding.40 Thus, while mathematical models are insightful and may prove useful for offering predictions, experimental verification of these mathematical models is also essential. Further, multiple bactericidal mechanisms are possible and may not be mutually exclusive.

Observational non-mechanistic studies provide direct evidence of the efficacy of a treatment on a particular
bacterial strain. However, bacterial strains vary in size, stiffness, and metabolic processes. Due to these bacterial differences, observational studies require testing antibacterial treatments iteratively against multiple strains to produce a generalized inference of the real-world applications of any antibacterial approach. Experimental studies of the E. coli/nanotexture interaction mechanisms are thus needed to complement existing observational studies and mathematical models. Here, both conventional transmission electron microscopy (TEM) and liquid-phase TEM are implemented to observe the mechanism of bacterial death. Liquid-phase TEM allows for high-resolution nanoscale observations of the E. coli/nanotexture interactions without requiring preservation of the sample. E. coli K12 is well-characterized in literature as a model organism and was thus chosen for this investigation. In contrast to previous works on films or larger fibers, here poly (lactic-co-glycolic acid) (PLGA) microparticles (MPs) are nanotextured and are shown to have an antibacterial effect on E. coli in liquid media. PLGA is biocompatible, biodegradable, low-cost, FDA-approved, and may be functionalized with other antibacterial treatments to produce a combined antibacterial effect if desired. Suspensions containing E. coli and nanotextured PLGA MPs were imaged in TEM via both conventional TEM and liquid-phase TEM via graphene liquid cells (GLCs) to observe bacterial death and structural changes in E. coli.

Materials and Methods

Bacteria Culturing

E. coli K12 were commercially purchased (Escherichia coli (Migula) Castellani and Chalmers, ATCC® 29425™). Starter cultures were preserved in 50% glyceraldehyde and 50% phosphate buffered saline (PBS) and frozen at −80°C as previously described. E. coli was cultured in 50mL lyso-geny broth (LB) at 37.5°C on an orbital shaker at 100RPM as previously described.

PLGA Microparticle Etching

PLGA MPs were commercially purchased (Degrdex® PLGA microspheres, LG500). The particles varied between several nanometers to several micrometers in diameter. The PLGA MPs were then dispersed in DI water and sonicated for 5 mins. The PLGA MP suspension was then dropcast in 10μL quantities onto approximately 5x5cm copper sheets and allowed to dry completely for at least 12 hrs. The PLGA-covered copper sheets were then plasma etched via the method described by Serrano et al. The particles were etched using a South Bay PC150 plasma etcher under 200 ppm oxygen at 100 W in 1 min increments, with 2 mins between each increment to prevent heating of the sample. After etching, the copper sheets were cut into strips and placed in microcentrifuge tubes containing 1 mL sterile PBS and sonicated for 5 mins to remove the PLGA MPs from the copper sheets.

SEM of PLGA MPs

PLGA MPs on copper after plasma etching were gold sputter-coated for 1 min and imaged in SEM via a RAITH100 eLine EBL at 2kV to 3kV with working distances of 13 mm to 20 mm.

AgNP Solution Preparation

Silver NPs (AgNPs) were commercially purchased and added to sterile PBS. The suspensions were then sonicated to facilitate suspension of the AgNPs, and exposed to UV light to sterilize the sample.

Colony Counting

E. coli were cultured in LB broth to the stationary phase of E. coli growth. Ten microliters were then extracted from each E. coli culture to determine viability prior to the addition of antibacterial materials. PLGA MPs and AgNPs were added to the appropriate E. coli samples, which were then immediately returned to the incubator. The extracted samples were diluted to 10⁻², 10⁻⁴, and 10⁻⁶, and plated on LB agar plates, as described previously. The colony counting procedure was repeated at 4 hrs and 8 hrs after the addition of the antibacterial or control samples to the E. coli culture to observe bacterial death over time. This procedure was repeated six times to produce statistically valid data. The results were analyzed via Student’s t-test.

Conventional TEM Imaging

E. coli was cultured to the stationary phase at which point PLGA MPs etched for 2 mins were added, incubated an additional 20 mins, and then fixed for imaging with conventional TEM. The stationary phase refers to the phase of bacterial population growth at which the total population is constant. Bacteria and the antibacterial agent were centrifuged to isolate the sample as a pellet, with the LB broth removed. The samples were then preserved in resin via glutaraldehyde and osmium fixing and staining, as per Feng et al. This conventional TEM method includes microtoming the samples to produce cross-sectional 2D slices for imaging.
circular due to the orientation of these bacteria within the 2D slice. A JEOL 1220 TEM was used for image acquisition. TEM images were false-colored for clarity. The original, unaltered images are included in the SI.

In situ TEM Imaging

*E. coli* was cultured to stationary phase in LB broth. PLGA MPs etched for 2 mins were added to the solution and incubated for an additional 20 mins. The LB broth was then removed via centrifugation and the sample rehydrated in PBS. Approximately 1 μL of the solution was placed on a graphene-coated TEM grid and then covered with another graphene-coated grid to produce a graphene sandwich. Textor et al provided a detailed review of this GLC synthesis process. The incident electron dose varied between 3×10⁹ Gy/s to 6×10¹⁰ Gy/s throughout the experiment. A JEOL 1220 TEM was used for imaging. TEM images were false-colored for clarity. The original, unaltered images are included in the SI.

Results and Discussion

In the first step, the PLGA MPs were plasma-treated to produce nanotextures on their surface. The MPs were plasma etched for 1, 2, and 5 mins to induce different surface roughness (see Material and Methods, Figure 1A–C). The PLGA MPs were imaged via SEM to observe the surface topology (Figure 1D–G). The PLGA MPs show minimal nanotexturing in particles etched for 1 min, while at 2 mins these nanotextures are deeper and more pronounced. At 5 mins of plasma etching, the sample is highly damaged leaving behind damaged, disordered PLGA. Particles etched for 2 mins, with the roughest surface morphology, were selected for antibacterial testing.

![Schematic of plasma etching of PLGA MPs (A-C), SEM imaging of the control PLGA MPs and the plasma-etched PLGA MPs (D-G) and viability graph of *E. coli* control and in the presence of antibacterial agents (H). In (A) oxygen flows in low concentration (200 ppm volume) through a charged environment to create free radicals (B) which etch the surface, leaving behind rough MPs (C). In (D) unetched particles show smooth surface morphology. In (E) the PLGA MPs show mild dimpling of the surface after 1 min of plasma etching. In (F) the PLGA MPs etched for 2 mins show more pronounced, sharp patterns as opposed to the PLGA MPs shown in (D) and (E). Finally, in (G) 5 mins of etching severely damaged the MPs, reducing the PLGA to primarily amorphous PLGA aggregations. The bactericidal efficacy of PLGA MPs and AgNPs are compared in (H) where control and bacteria treated with 3 μg/mL AgNPs do not show decreased CFU/mL, while the 100 μg/mL AgNPs samples show decreased viability. The error bars in (H) represent the standard error. The unetched PLGA does not exhibit a statistically significant bactericidal effect, nor does the PLGA etched for 5 mins. However, the PLGA etched for 2 mins, which features rough MP surfaces, does exhibit a statistically significant bactericidal effect. The scale bar is 1 μm in (D), and 500 nm in (E), (F), and (G).
In the next step, the bacterial activities of treated and untreated PLGA MPs were studied and compared with AgNPs. Unetched smooth PLGA MPs, PLGA MPs etched for 2 mins, and PLGA MPs etched for 5 mins were added in concentrations of 6 μg/mL to separate E. coli cultures at the stationary growth phase (see Material and Methods, Figure 1H). Additionally, AgNPs, known to have bactericidal effects, were added to separate LB broth solutions in concentrations of 3 μg/mL AgNPs and 100 μg/L AgNPs to create positive controls (see Material and Methods). The results show the PLGA MPs etched for 2 mins lowered the viability of E. coli by approximately 64% (p<0.05) as compared to controls, while the 100 μg/mL AgNPs exhibited bactericidal effects which lowered the viability of E. coli by approximately 57% (p<0.10) (Figure 1H). The E. coli sample containing 3 μg/mL AgNP may not have shown a statistically significant antibacterial effect (Figure 1H) due to the large size of the AgNPs used in this work, which may reduce the bactericidal efficiency of AgNPs as compared to smaller AgNPs used in other studies (Figure 1H). The control, smooth PLGA MPs did not kill E. coli, in agreement with previous findings that PLGA is non-toxic. The highly damaged amorphous PLGA MPs likewise do not exhibit any bactericidal effect, suggesting that the plasma etching process does not alter the chemistry or surface properties of the PLGA MPs to produce bactericidal effects. Further, the increased surface area on the highly damaged PLGA etched for 5 mins would be expected to increase the bactericidal effect if bacterial death was due to a chemical effect rather than the rough surface topology. Only the PLGA MPs etched for 2 mins with the rough nanotextured surface kill bacteria, to which only the nanotextures are unique, thus indicating that it is the nanotextures that kill E. coli.

Direct mechanical damage to the cell wall compromises the cell envelope permeability and induces cell death. Metabolically mediated cell wall degradation may occur due to a cessation of metabolic cell wall maintenance or by the production of intracellular autolysins which actively break down the cell wall. Further, escape of the bacterial cytosol, the fluid within bacteria, and a decrease in volume are indicative of bacterial death. Conventional light microscopy therefore uses fluorescent dyes to determine the integrity of the cell wall regardless of the mechanism of bacterial death. However, TEM allows nanoscale observation of the cell envelope and MPs and thus does not require fluorescent dyes since cell-wall damage can be directly deserved.

Nanotextured PLGA MP/bacteria surface interactions were examined via conventional TEM as shown in Figure 2 (see Material and Methods) and Figure S1. In this figure, a PLGA MP has rough nanotexture and uneven edges as observed with SEM (Figure 1). As shown in Figure 2A and B (progressive magnification) (also in Figure S1), the MP has a surface protrusion inside an adjacent bacterium. The location of the surface protrusion suggests that the MP sharp features pierced the cell envelope of the bacterium, including the outer membrane, peptidoglycan layer, and inner membrane. Piercing the cell envelope would produce local cell damage and lysis of the bacterium. The absence of the bacterial cell envelope in Figure 2B indicates that this damage was sufficient to stop the metabolic maintenance of the cell envelope or produce apoptosis. In contrast to mathematical modeling in previous

![Figure 2](https://example.com/figure2.png)

**Figure 2** Bacterial death is induced by rough MP topography as observed in a cross-sectional image obtained via conventional TEM. In (A) and (B) low to high magnifications of surface interaction between an E. coli and a rough PLGA particle etched for 2 mins is shown. A sharp peak on the PLGA particle appears to have penetrated the E. coli bacterium cell envelope. The area bracketed in (A) is shown at higher magnification in (B). The scale bars in (A) and (B) are 200nm.
works, in Figure 2 the E. coli is not stretched between nanopillars but rather is in contact with a single sharp protrusion, which appears to have been directly penetrated the cell surface (Figure S1).  

E. coli and PLGA MPs etched for 2 mins were then encapsulated together in GLCs (Figures 3 and S2). In GLCs, a liquid sample is encapsulated between two layers of graphene (see Material and Methods).  

The graphene layers are impermeable to liquid matter, fully encasing the sample, and allow transmission of the electron beam through the sample. The graphene also dissipates energy, which reduces undesired effects of the beam exposure (SI, Liquid-TEM Imaging).  

Previous works have shown that the effects of changes in pressure, temperature, free radical generation, and exposure to the electron beam itself are non-significant under low electron doses for large samples, such as those containing E. coli, as implemented here (SI notes). Thus, previous works indicate that E. coli 1) maintain their metabolic processes, 2) maintain their cell envelopes, and 3) continue to undergo binary fission within liquid-phase TEM. 

Liquid-phase TEM (Figures 3 and S2) provides further evidence of the penetration of nanoprotrusions in E. coli, as also observed forensically with conventional TEM (Figures 2 and S1). The cell envelope of the bacterium immediately adjacent to a rough PLGA MP is highly damaged and shows escape of the bacterium’s cytosol. In contrast, areas distal to the PLGA MP surface protrusion show smooth, undamaged cell envelopes. The localization of cell damage to the site of bacterium/PLGA MP interaction indicates that the bacterium was not killed by general metabolic factors, which would cause diffuse damage throughout the bacterium cell envelope. Bacterial death thus must have occurred due to the localized damage produced by the E. coli/surface interaction. 

Gram-negative bacteria, including E. coli, contain peptidoglycan cell walls approximately 2nm to 8nm in thickness. In contrast, gram-positive bacteria have cell wall thicknesses from approximately 20nm to 35nm. Peptidoglycan has been considered to provide the structural support and is a primary contributor to the mechanical properties of bacteria. The thickness of the peptidoglycan layer might be intuitively expected to reflect the stiffness of bacteria. In contrast to this expectation, initial experimental studies reported that gram-negative bacteria exhibit an average Young modulus of approximately 30MPa while gram-positive bacteria exhibit a Young modulus of 20MPa. Further studies showed that the Young modulus varies significantly depending on the bacterial species, growth medium, preparation method, dry or wet state, and measurement method, with previously reported Young modulus values between 0.05MPa and 769MPa. This variation in mechanical properties reflects cell wall variations in proteins, phospholipids, teichoic acids, lipoteichoic acids, and differences in peptidoglycan-peptidoglycan binding or interactions. Peptidoglycans consist of alternating sugar moieties, β-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid, which are then attached to a peptide chain. While the sugar moieties are highly conserved between bacterial species, the attached peptide chains are bacterial strain-specific and vary significantly in peptide chain length and amino acid composition. 

Figure 3 GLC encapsulation of E. coli and PLGA MPs shows localized damage to the cell envelope of the bacterium. In (A), a lower-magnification image shows an overview of the E. coli and PLGA MP. In (B), a higher magnification shows the PLGA MP and bacterium in close proximity. The cell wall proximal to the PLGA MP shows damage with a shape similar to the adjacent MP. A high contrast liquid indicates the cytosol of the bacterium, which clearly identifies the death of the bacterium. The cytosol has a distinct contrast from the PBS medium due to the proteins, glycans, and other bacterial components within it. Other areas of the E. coli cell wall are smooth and show no damage or degradation. The scale bars in (A) and (B) are 200 nm.
Peptidoglycans may be monomers or cross-linked as dimers or trimers, with linkages between a variety of amino acid residues.\textsuperscript{83,84} The variation in peptide chains and the activity of crosslinking proteins produces unique chain crosslinking.\textsuperscript{85,86} The degree and type of peptidoglycan crosslinking are thus highly positively correlated with the stiffness of the bacteria.\textsuperscript{80} Some bacteria may also contain actin-like filaments that form a rudimentary cytoskeleton.\textsuperscript{87} These actin-like filaments may influence the mechanical properties of bacteria, including their stiffness.\textsuperscript{87} In short, the thickness of the peptidoglycan layer and the gram-positive/gram-negative classification are not sufficient to establish the mechanical properties of bacteria.\textsuperscript{41} This suggests that the bactericidal nature of nanoscale surface topologies may also be effective in gram-positive bacteria, since the cell wall of such bacteria may be comparable to \textit{E. coli}.\textsuperscript{41}

The deformation of the bacterial cell wall thus depends not on simple physical forces and the thickness of the peptidoglycan layer, but on the Young modulus of the bacterial cell wall and the attachment forces of the bacterial adhesion proteins. \textit{E. coli} was previously shown to exhibit a Young modulus of 22 MPa.\textsuperscript{88} This suggests the attachment pressure of \textit{E. coli} exceeds this value to produce strain within the cell wall to produce the damage shown in Figures 2 and 3 (Figures S1 and S2). The bactericidal mechanism of antibacterial nanotextures observed here and supported by literature is illustrated schematically in Figure 4. First, simple physical forces, eg, hydrophobic/hydrophilic interactions, bring the \textit{E. coli} into contact with the PLGA particle surface.\textsuperscript{38,39} The adhesion proteins then attach and contract to bind the \textit{E. coli} to the PLGA surface.\textsuperscript{38,39} However, the protruding fixture on the PLGA surface exerts a local force opposing the adhesion proteins bound to the non-protruding surface (Figures 2, 3 and S1, S2). This causes stretching in the \textit{E. coli} cell membrane over the PLGA nanopillar, as experimentally observed here in Figures 2 and 3 (Figures S1 and S2) and illustrated in Figure 4A–D, which damages the cell envelope. This damage causes the death of the \textit{E. coli} and leakage of the cytosol localized to the nanotexture projection, as experimentally observed in Figures 3 and S2. This cell

![Figure 4 Schematic illustration of bacterial death mechanism. The bacterium is shown in blue on the left side of the image (i), whereas the cell envelope is indicated as a dark blue layer on the outer edge of the bacterium (ii). The PLGA particle is shown in grey on the right-hand side (iii). In (A) and (B) the particle has come into contact and deforms the cell envelope, before breaking it in (C) and (D). This damage then causes degradation of the cell envelope in (E) and (F), before the cell wall disintegrates in (G) and (H).](image-url)
death later results in total degradation of the cell envelope due to reduced metabolic cell envelope maintenance or production of autolysis, as experimentally observed in Figure 2 (Figure S1) and illustrated in Figure 4E. Here, a mechanism by which nanoscale surface roughness may kill E. coli K12 has been shown.

Conclusions

We report rough nanotextured polymer MPs that exhibit antibacterial action. Such MPs have applications distinct from previously designed media, such as antibacterial gels, liquid suspensions, or powders. The nanotextured surface topology of the MPs was shown to be bactericidal via a localized bacterial action. Such MPs have applications distinct from previously designed media, such as antibacterial gels, liquid suspensions, or powders. The nanotextured surface topology of the MPs was shown to be bactericidal via a localized E. coli/surface interaction mechanism. The MP surface protrusions deform bacterial cell walls to induce bacterial death rather than act by damaging bacterial cell walls via stretching between nanopillars. This cell-wall damage suggests that the attachment pressure exceeded the force required for deformation of the cell wall of the bacterium. The deformation of the cell envelope appears to cause cell lysis and death of the E. coli. Future studies are required to explore the nanoscale interaction of rough surface topology with other types of bacteria, and to further exploit the antibacterial mechanism suggested here. One should be aware that the success rates of liquid-phase TEM experiments are very low. The reproducibility of such liquid-phase TEM tests is also very challenging and needs to be revisited. We believe more careful studies are needed to ensure the bacteria are alive in the TEM environment.

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Disclosure

The authors report no conflicts of interest in this work.

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