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Electrically conductive bacterial nanowires in bisphosphonate-related osteonecrosis of the jaw biofilms

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Objective. Bacterial biofilms play a role in the pathogenesis of bisphosphonate-related osteonecrosis of the jaw (BRONJ). The purpose of this preliminary study was to test the hypothesis that the extracellular filaments observed in biofilms associated with BRONJ contain electrically conductive nanowires.

Study Design. Bone samples of patients affected by BRONJ were evaluated for conductive nanowires by scanning electron microscopy (SEM) and conductive probe atomic force microscopy (CP-AFM). We created nanofabricated electrodes to measure electrical transport along putative nanowires.

Results. SEM revealed large-scale multispecies biofilms containing numerous filamentous structures throughout necrotic bone. CP-AFM analysis revealed that these structures were electrically conductive nanowires with resistivities on the order of 20 Ω·cm. Nanofabricated electrodes spaced along the nanowires confirmed their ability to transfer electrons over micron-scale lengths.

Conclusions. Electrically conductive bacterial nanowires to date have been described only in environmental isolates. This study shows for the first time that these nanowires can also be found in clinically relevant biofilm-mediated diseases, such as BRONJ, and may represent an important target for therapy. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:71-78)

More than 40 million Americans and more than 200 million patients worldwide are currently receiving antiresorptive drugs to treat common bone disorders such as osteoporosis and skeletal complications associated with osseous metastasis and multiple myeloma.[1] Osteonecrosis of the jaw is a serious adverse effect associated with bisphosphonate (BP) antiresorptive therapy. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is characterized by necrotic jaw bone in the oral cavity. The pathogenesis of BRONJ is considered to be multifactorial and usually involves oral trauma, with subsequent delayed wound healing and a biofilm-mediated infection.[2,3] Spontaneous cases of BRONJ have been reported without mucosal breach, although in most cases patients who develop BRONJ have a history of invasive dental procedures or complications from tooth extractions or denture trauma that places them at risk for BRONJ.[4] Invasive oral surgical procedures or trauma to jaw bone can expose bound BP from the bone, releasing the drug into the local milieu, where it has been shown to inhibit wound healing and increase the binding affinity of oral bacteria to bone, culminating in BRONJ.[5] Sedghizadeh et al. described complex microbial communities that permeate the affected jaw bone structure, implicating biofilms in the mineralogic dissolution of bone by yet unknown mechanisms.[6] The types of bacteria described in BRONJ biofilms are difficult to treat and have known resistance to conventional antibiotics.[7] Innovative approaches that destabilize the structural or metabolic integrity of biofilms are needed to improve our ability to treat BRONJ and other biofilm-mediated infectious diseases. Such innovations require a more complete understanding of the processes that sustain microbial biofilms so that therapy could target the disruption of these processes.

BRONJ biofilms, like many clinically relevant biofilms, produce extracellular filaments that represent a large fraction of the extracellular biofilm matrix. Filamentous extracellular structures in biofilms have commonly been classified as part of the extracellular polymeric substance (EPS), which includes polysaccharides, flagella, DNA, RNA, and a variety of dissolved small compounds.[8-11] Recent evidence has revealed that a variety of microorganisms produce electrically conductive filaments called bacterial nanowires that appear to be important contributors to extracellular electron transfer.[12,13] Bacterial nanowires were first described in 2005 as a phenomenon occurring in metal-reducing bacteria.[14] They were implicated in the transfer of electrons to solid-phase electron acceptors, such as iron and manganese

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Table I. Clinical-pharmacological characteristics of study patients

Patient # age/sex	*Ethnicity*	*BP type/duration*	*BP indication*	*Location of BRONJ*	*Dental factor*	*BRONJ stage*
1. 67/M	Hispanic-American	Pamidronate/36 months	Prostate cancer	Left maxilla	Tooth extraction	III
2. 68/M	African-American	Zoledronic acid/8 months	Multiple myeloma	Right mandible	Tooth extraction	II
3. 63/F	Hispanic-American	Alendronate/36 months	Osteoporosis	Right maxilla	Denture trauma	II
4. 76/F	Asian-American	Alendronate/120 months	Osteoporosis	Left mandible	Tooth extraction	II

oxides. Importantly, unique nanofabrication techniques combined with imaging techniques such as conductive probe atomic force microscopy (CP-AFM) were necessary to prove that bacterial nanowires had actual electrical or conductive properties. Conductivity is the measure of the ease at which an electric charge can pass through a material. Before the demonstration of conductivity, structures could not be uniquely classified as bacterial nanowires, because they could merely represent some known component of biofilm EPS. Confirmation of conductive nanowires in a variety of other environmental systems, including an oxygenic photosynthetic cyanobacterium and a thermophilic methanogenic coculture, suggested early on that nanowires might be common structures in diverse microbial systems; most published research describing the conductive properties of bacterial nanowires has focused on the environmental metal-reducing bacteria *Geobacter sulfurreducens* and *Shewanella oneidensis* MR1.[15,16] The complete composition and role of bacterial nanowires remains largely unknown and unexplored, because bacterial nanowires are a recent discovery and researched by only a few laboratories. To date, the work on bacterial nanowires has focused on environmental microbes, and nanowires have not been identified or characterized in human pathogens.

The exact physiologic role of bacterial nanowires remains to be elucidated, but the nanofabrication and imaging techniques used for evaluating the conductive properties of these biologic protein nanostructures are now well established. These techniques have been applied to the conductive or electromicrobiologic characterization of environmental bacteria but not to medically and clinically relevant microbes. Therefore, the purpose of this preliminary study was to apply nanofabrication techniques along with CP-AFM imaging techniques to test our hypothesis that the extracellular filaments observed in microbial biofilms associated with BRONJ contain electrically conductive nanowires.

MATERIALS AND METHODS

Patient sample collection

Samples of necrotic bone were collected from patients as part of an ongoing natural history study at the University of Southern California. Before sample collection, appropriate Institutional Review Board approval (no. HS-CG-05-00002) and written patient consents were obtained. Samples were obtained from 4 patients requiring routine clinical sequestrectomy procedures as previously described,[17] and study patient characteristics are provided in Table I. For study inclusion, all patients required a diagnosis of a stage 1-3 BRONJ lesion, established by standard clinical and radiographic protocol per the American Association of Oral and Maxillofacial Surgeons diagnostic criteria.[18] To minimize confounding variables or effect modifiers, patients were excluded from the study if they had a history of head and neck radiation or concomitant steroid therapy, because these can cause osteonecrosis unrelated to BP therapy. One sample of bone from each patient affected by BRONJ in our study was cut into subfragments and prepared for microscopic and electrical conductivity analyses as described below.

Scanning electron microscopy

Scanning electron microscopy (SEM) allows for microscopic evaluation of specimens at magnifications over a range of up to 6 orders of magnitude (×10,000-500,000), about 250 times the magnification limit of the best light microscopes. This makes SEM an ideal technology for evaluation of microscopic biofilms which could be missed via routine histopathologic evaluation. For SEM analysis in our study, fragments of bone samples from every study patient were fixed in a 4% formaldehyde/2% glutaraldehyde solution for 48 hours at 4°C, washed with phosphate-buffered saline solution buffer, dehydrated in a graded ethanol series, critical point dried, and mounted on an aluminum stub. The samples were then sputter coated with platinum (Pt) and imaged with the SEM operating at 5 kV in the secondary electron mode (XL 30S; FEG, FEI Co., Hillsboro, OR).

Conductive probe atomic force microscopy

CP-AFM allows for current/voltage (I/V) measurements by applying voltage to putative electrical structures and measuring the current along the wire as the voltage changes. Typically, a linear to nonlinear response is expected for the resulting I/V data at some negative to positive value of current if the structure has conductive properties, otherwise a flat horizontal line at zero current results, which would indicate lack of conductivity. For CP-AFM analysis, fragments of each bone sample were mechanically disrupted with sterile 18-gauge needles, and 20 mL of the suspension was spotted onto highly ordered pyrolytic graphite (HOPG) wafers (SPI Supplies, West Chester, PA). The HOPG wafers act as a flat and electrically conductive substrate for the biofilms, facilitating the conductive probe measurements with CP-AFM. For the purposes of our study, CP-AFM was used in 2 common modes: tapping mode and contact mode. Tapping mode is used to map topography of a surface (e.g., HOPG biofilm sample) by applying an oscillating cantilever probe tip to a surface via light tapping. The cantilever's oscillation amplitude changes with sample surface topography, and a topographic image is obtained by monitoring these changes. In contact mode, the probe tip is in perpetual contact with the sample, in contrast to tapping mode, and conductivity measurements are made in contact mode. The HOPG biofilm samples were allowed to air dry until all of the fluid had evaporated. Then, they were gently washed with distilled H_2O delivered dropwise before air drying again. CP-AFM was performed with the use of a Dimension V Scanning Probe Microscope (Veeco Instruments, Plainview, NY). The samples were first imaged in tapping mode, using NSC36/AlBS probes (Mikromasch, San Jose, CA) to obtain topographic images of the sample. Once areas with putative nanowires were located, the probe was replaced with a Pt-Iridium–coated conductive contact tip (SCM-PIC; Veeco Instruments) for the conductivity measurements in contact mode. For control, an open circuit was created by placing 2 Pt probes very close together (<150 nm without a bridging nanowire) on subsamples that underwent the same SEM and CP-AFM protocol.

Nanofabrication technique for measuring electron transport and resistivity

For electrical transport measurements, bone samples were deposited onto oxidized silicon wafers with prefabricated gold electrodes and loaded into a Zeiss 1540 FIB/SEM electron microscope (Carl Zeiss Microscopy, Thornwood, NY). As described by our group previously,[12] putative nanowires in the uncoated samples were located by SEM in the proximity of the prefabricated gold contacts. Gold is used in this technique because it ensures sufficient contact and is a well established conductor, like silver or copper, but does not readily oxidize. Once a suitable nanowire was found in each sample, Pt precursor gas was injected into the chamber in close proximity to the sample surface. Pt leads that contacted the nanowires were deposited by patterned exposure to the electron beam. Longer Pt leads (e.g., to bridge the connection to the gold electrodes) were established by exposure to the ion beam. I/V measurements were performed at room temperature using a probe station instrumented to an Agilent 4156C (Agilent Technologies, Santa Clara, CA) semiconductor parameter analyzer. Nanowire resistivity (ρ) was calculated as $\rho = RA/L$, where R is the measured resistance, A is the cross sectional nanowire area (calculated with AFM height measurements described above), and L is the length of the nanowire segment between the 2 probes measured by SEM and CP-AFM imaging.

RESULTS

Scanning electron microscopy

SEM analysis of BRONJ-affected bone from all 4 patients revealed thick multispecies biofilms permeating the bone tissue and composed of predominantly bacterial morphotypes (Figure 1). All four of the study patients had necrotic bone colonized by biofilms with 15 distinguishable bacterial morphotypes similarly observed in each specimen. Morphotypes included organisms from the genuses *Fusobacterium*, *Bacillus*, *Actinomyces*, *Staphylococcus*, *Streptococcus*, and *Selenomonas* and 3 different morphotypes of treponemes. The bacteria identified in all 4 of the specimens were similar at the SEM level, and comprised gram-positive and gram-negative organisms with predominantly anaerobic and facultative anaerobic organisms. The biofilms were associated with large portions of necrotic bone tissue and some areas of the samples appeared to contain more microbial biomass than bone. Many thin filaments (e.g., putative nanowires) were seen connecting bacteria (Figure 1, *B* and *C*). There were no major differences that could be observed between the nanowires among the various subsamples. Based on morphologic similarities between these structures and nanowires produced by environmental organisms, we applied our nanofabrication approach with CP-AFM imaging for testing whether some of these filaments were conductive nanowires. Although it is quite likely that the EPS contains a combination of pili, flagella, polysaccharides, and DNA, nanofabrication and imaging techniques were applied to test our hypothesis that some of these filaments are truly conductive bacterial nanowires.

CP-AFM

CP-AFM was used to determine if the cellular appendages observed in the SEM micrographs were conductive nanowires. Figure 2 shows a tapping-mode topographic scan of an elongated bacterium with numerous filamentous appendages protruding from the cell. Panel *A* shows a deflection image giving a 3D image, and

Fig. 1. **A,** Clinical photograph of patient no. 3, demonstrating a typical BRONJ lesion involving the maxillary ridge with characteristic nonhealing mucosa (center of image) and exposed and infected sequestrum evident at the base of the ulcer. **B,** SEM photomicrograph of bone from patient no. 2, showing the multispecies biofilms characteristic of BRONJ (original magnification ×1,000). **C,** SEM photomicrograph of bone from patient no. 3, showing the filamentous structures connecting the cells (original magnification ×5,000). **D,** In this routine hematoxylin-eosin–stained histologic section of the infected bone from patient no. 1, bacterial biofilms are not as identifiable as the in the SEM images, given the lower magnification of light microscopy (original magnification ×60), but bacteria-like morphotypes can be seen attached to the subjacent surface of the nonvital bone in association with inflammatory cells and erythrocytes.

panel *B* corresponds to a topographic image. An I/V measurement was obtained by placing the AFM tip at the position marked by the blue triangle and sweeping the voltage from −1 to +1 V. The resulting I/V curve as shown in Figure 2 demonstrates a conductive nonlinear response to the applied voltage. Lack of conductivity on an I/V curve would show as a straight horizontal line at zero current, which was seen for control samples, as described later; therefore these results confirm conductivity of the identified structures and their designation as bacterial nanowires. Further imaging of bone sections from all patient samples revealed numerous bacteria with extracellular filamentous appendages. One example was a long nanowire with multiple branches as shown in Figure 3. Although the width of the nanowire is exaggerated by the radius of curvature of the AFM probe, the topographic data show it to be ~5 nm in length (Figure 3, *A*). Figure 3, *B*, shows the current measured at each point of the scan with 100 mV applied between the HOPG substrate and the Pt-Iridium–coated AFM probe, confirming that both the branches and the main trunk of this nanowire are electrically conductive. A corresponding I/V curve taken from a point along the nanowire showed a nonlinear response similar to that measured in Figure 2. The original tapping-mode scans of the nanowire in Figure 3 showed it to be covered in a relatively soft blanket of material (Figure 4, *A*), presumably exopolymers and other suspended materials that were deposited along with the nanowires when the sample was air dried. After switching from tapping-mode AFM to contact mode, this material was pushed aside by the AFM probe, "cleaning" the nanowires as shown in the bottom of Figure 4, *B*, and allowing for high-resolution conductivity measurements. Control samples showed no current response to applied voltage, ruling out any metallic contamination between the electrodes under the deposition conditions used in this study.

Nanofabrication technique for measuring electron transport and resistivity

Figure 5, *A*, shows a nanowire contacted by platinum leads. Each lead is connected to a gold electrode as before (not pictured), which was used to apply voltage and measure the current along the nanowire as shown in Figure 5, *B*. We measured the length of this wire to be 500 nm and the diameter 5 nm, and thus assuming a relatively circular cross section, the resistivity of the nanowire was calculated to be ~20 Ω·cm.

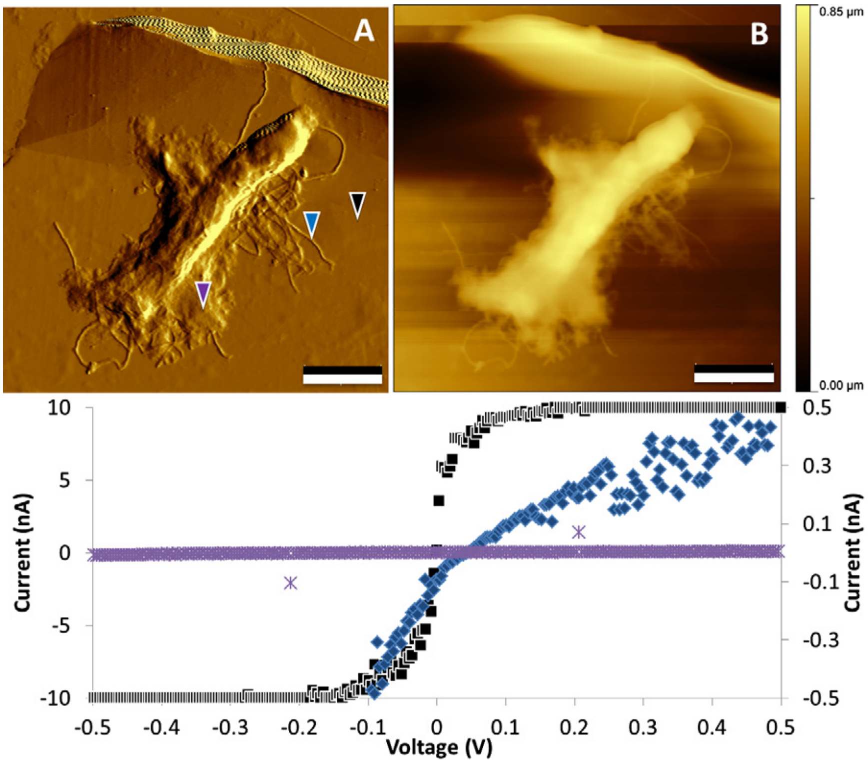

Fig. 2. **A,** Tapping-mode amplitude and **B,** topographic images of a bacterial cell from BRONJ-affected bone of patient no. 3 dried onto an HOPG surface (*black arrowhead*). Putative nanowire filaments are readily observed radiating from the cell (*blue arrowhead*). The cell is also surrounded by other EPS material (*purple arrowhead*) that is morphologically distinct from the nanowires. The graph shows the current/voltage (I/V) plot obtained with the AFM in contact mode and the probe tip placed on a nanowire at the position marked by the blue arrowhead. The displayed I/V data were averaged over 10 voltage sweeps. The control showed no conductivity, as represented by the horizontal straight purple line at zero current.

DISCUSSION

BRONJ pathogenesis is thought to be multifactorial, yet one of the clinical features consistently associated with this condition is a biofilm-mediated infection.[2] Although spontaneous BRONJ cases have been reported, most cases occur after invasive dental procedures or oral trauma to bone, resulting in bone exposure and colonization by oral biofilm pathogens.[3] Therefore, an understanding of the pathophysiology of BRONJ biofilms is important for informing targeted therapeutics in the future. The biofilm lifestyle is preferred by most bacteria in natural environments, including those within the human body.[19,20] The biofilm matrix consists of not just cells but a complex collection of cells, extracellular appendages, extracellular proteins, carbohydrates, and nucleic acids.[21] The extracellular component of this matrix, the EPS, has proposed functions such as aiding in adhesion in the early stages of biofilm formation, providing a barrier to desiccation and antimicrobial agents, and creating distinct redox microenvironments.[10] Extracellular appendages, such as type IV pili and flagella, play an important role in the ultrastructure of mature biofilms, such as those observed in the model biofilm organism *Pseudomonas aeruginosa*.[8] Bacterial nanowires, such as those reported in the present study, appear to be a specialized subset of extracellular appendages that serve to both stabilize the biofilm and facilitate the transfer of electrons within the biofilm, potentially contributing to biofilm survival, proliferation, and pathogenicity.

Bacterial nanowires have been observed in environmental microbiology and characterized in environmental isolates ranging from metal-reducing bacteria such as *S. oneidensis* and *G. sulfurreducens* to the phototrophic cyanobacterium *Synechocystis* species PCC6803.[15,16] The variety of ecologic niches occupied by these organisms and their differing metabolic strategies suggest that nanowires are important in many natural microbial habitats. The present study is the first demonstration of conductive bacterial nanowires within a clinically relevant microbial community. The human oral microbiome, an area of extensive research, is dominated by *Actinobacteria*, *Bacteroides*, *Fermicutes*, *Proteobacteria*, and many others.[22] The vast diversity of species within the human oral microbiome makes it difficult to pinpoint the exact species producing the

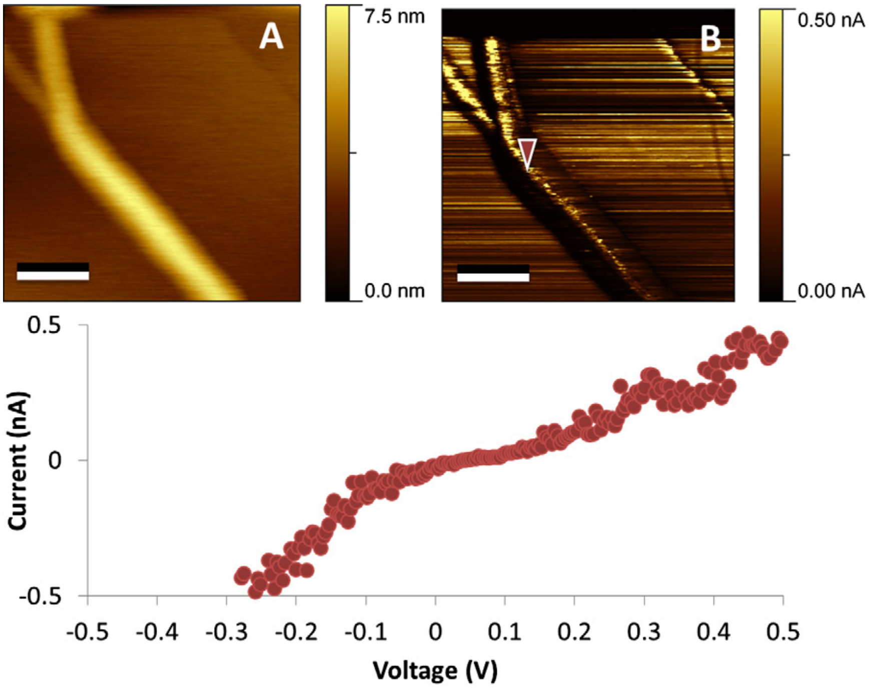

Fig. 3. **A,** Topography of a branched filament obtained by CP-AFM. **B,** Conductivity measurements of the nanowire shown in panel *A*, with 100 mV applied between the tip and sample. The graph shows I/V plot obtained with the AFM tip placed on the region of the nanowire marked by the red arrowhead.

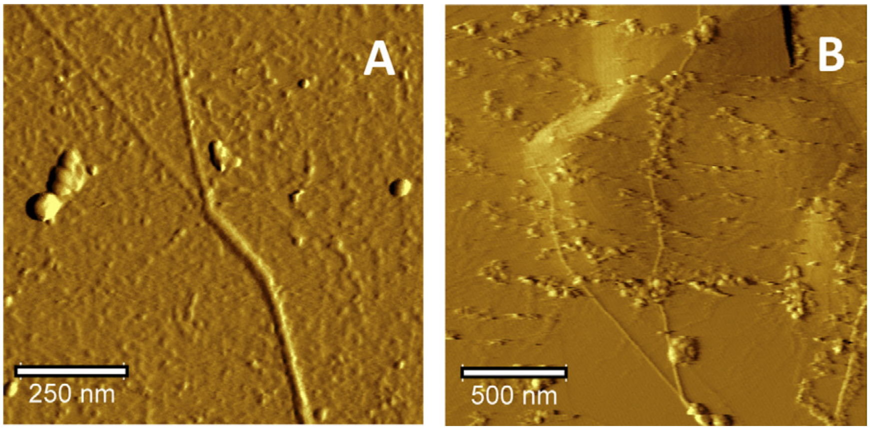

Fig. 4. **A,** Tapping-mode deflection image of the nanowire shown in Figure 3 before switching to conductivity mode. **B,** Contact-mode deflection image of the area surrounding the nanowire in panel *A*. After the scans in contact mode, the loose material blanketing the nanowire was removed, facilitating higher-resolution CP-AFM.

nanowires in the present study, which is a limitation that will need to be addressed in future investigations.

The physiologic and ecologic significance of electrically conductive bacterial nanowires is poorly understood. Nearly all of the published research on these structures has focused on metal-reducing bacteria.[14-16] Understandably, nanowires from these organisms were implicated in the transfer of electrons to solid-phase iron or manganese oxides and to electrode surfaces in microbial fuel cells. Although little is known regarding the physiologic roles and the molecular composition of bacterial nanowires, the methods used to confirm and evaluate their conductive properties are well established in the microelectromechanical systems field. By applying these methods to diverse microbial systems, we seek to determine the breadth of their distribution throughout the microbial world in nature and in disease. Although the research presented here does not directly establish the roles of these conductive structures in BRONJ biofilms, it does support the hypothesis that

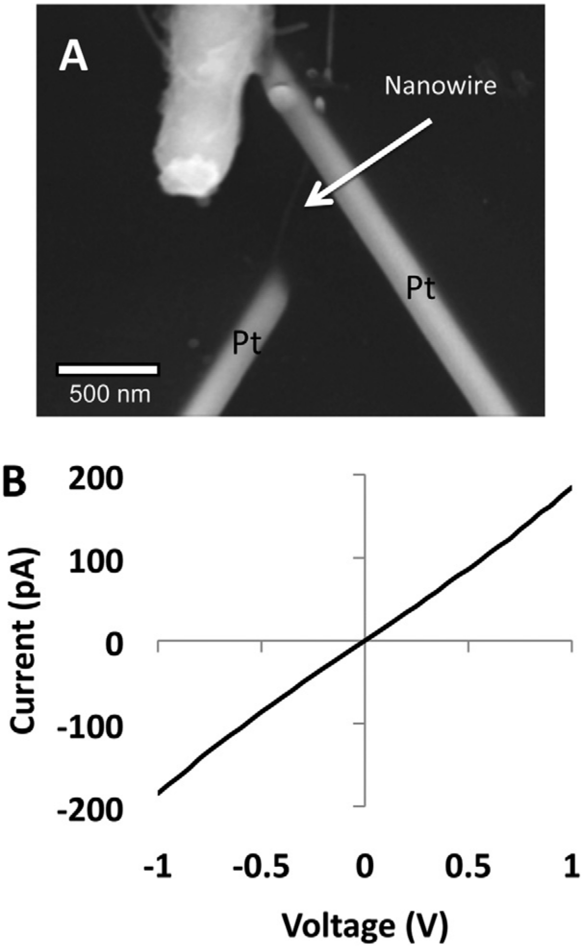

Fig. 5. **A,** SEM of platinum (Pt) leads contacting a bacterial nanowire on a silicon oxide surface. **B,** Current vs. voltage sweep obtained from the nanowire shown in panel *A*. The resistivity of the nanowire is ~20 Ω·cm.

conductive nanowires are common in diverse microbial systems and essential to biofilm survival.

The nonlinear current response observed in transverse conductivity measurements of the BRONJ-associated nanowires (Figures 2 and 3) is similar to the response observed in *S. oneidensis*.[13,14] Moreover, when conductivity is measured along the length of the nanowire (Figure 5), the response to the applied voltage is strongly linear, again consistent with measurements on *S. oneidensis*.[13,14] The resistivity value of ~20 Ω·cm obtained for BRONJ nanowires is similar to that of nanowires from *S. oneidensis*, where measured resistivities ranged from 1 to 17 Ω·cm.[13] Data such as these will help to determine the molecular mechanisms for electron transport along the nanowires. We also found that BRONJ nanowires are robust (Figure 4) and retain their structure throughout the scans, suggesting that they may incorporate a different structural component or that their overall composition may be substantially different from environmental nanowires. A recent study of the structural integrity of nanowires from *S. oneidensis* was able to determine the elastic modulus on the order of 1-4 GPa.[23] These values are similar to elastic moduli measured from peptide α-helices and are on the order of many man-made materials, such as nylon or polyethylene terephthalate.

One of the functions of the nanowires in BRONJ biofilms is presumably to transfer electrons through the biofilm. To respire, bacteria in the lower depths of the biofilm (near the bone tissue) require a conductive path by which to shuttle electrons to a terminal electron acceptor. Nanowires may facilitate the transfer of electrons through the biofilm, as has been shown in *G. sulfurreducens* biofilms grown on the anodes of microbial fuel cells.[24] Meanwhile, however, the buildup of protons or other metabolic acids in the depths of the biofilm would act to lower the pH of the surrounding biofilm and may limit biofilm growth. Cells growing on bone or the surface of a tooth have access to a solid substrate capable of removing the excess protons. If this model proves correct, disrupting bacterial nanowires could prove to be an effective strategy for compromising problematic biofilms and increasing their sensitivity to antibiotic treatment. It has been well established that biofilms stimulated with electric current display increased susceptibility to antibiotics, a phenomenon termed the "bioelectric effect."[25] Although the mechanism underlying this effect remains unknown, one possibility is that the electrical stimulation of these biofilms disrupts conductive nanowires, weakening the biofilm community as a whole. Another function of nanowires may be to allow for electrical communication between cells, analogous to the electrically conductive nervous system seen in vertebrate organisms.

Finally, the excessive resorption of the bone tissue seen in BRONJ can not be explained solely by osteoclastic activity or immunogenic destruction, especially considering that patients are on potent antiresorptive therapy. The mineralogic destruction of the bone tissue could be enhanced by the presence of active biofilms with electrically conductive nanowires. Bacterial nanowires are part of an electrical distribution system that facilitates electron transfer from the reducing environment within biofilms to electron acceptors located at more electropositive potentials near the biofilm surface. Solid-state charge transfer via conductive nanowires could establish charge separation and thereby contribute to thermodynamically unfavorable conditions in diffusion limited biofilms, with protons accumulating at the base. Calcium phosphate in bone (hydroxyapatite) could serve as a solid-phase pH buffer and consume protons as it dissolves. This will be an interesting theory to test in future lines of investigation in this field.

CONCLUSION

BRONJ is a relatively recently identified oral pathologic condition that is difficult to treat clinically because there is no established consensus on therapeutics or known cure. In the present proof-of-concept study, we have shown the presence of electrically conductive bacterial nanowires in BRONJ lesions. Bacterial nanowires may play an important role clinically in the pathogenesis of BRONJ biofilms. Although this phenomenon has been described in environmental microbiology, to our knowledge this if the first demonstration of conductive nanowires in medical or clinically relevant biofilms. Our findings suggest that electrically conductive nanowires may be ubiquitous in nature, and these nanowires may play important roles in biofilm homeostasis, communication, and pathogenesis, possibly representing a unique antimicrobial therapeutic target in the future.

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