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Imaging Hydrated Microbial Extracellular Polymers: Comparative Analysis by Electron Microscopy

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ABSTRACT

Microbe-mineral and -metal interactions represent a major intersection between the biosphere and geosphere but require high-resolution imaging and analytical tools for investigating microscale associations. Electron microscopy has been used extensively for geomicrobial investigations and although used bona fide, the traditional methods of sample preparation do not preserve the native morphology of microbiological components, especially extracellular polymers. Herein, we present a direct comparative analysis of microbial interactions using conventional electron microscopy approaches of imaging at room temperature and a suite of cryogenic electron microscopy methods providing imaging in the close-to-natural hydrated state. In situ, we observed an irreversible transformation of the hydrated bacterial extracellular polymers during the traditional dehydration-based sample preparation that resulted in their collapse into filamentous structures. Dehydration-induced polymer collapse can lead to inaccurate spatial relationships and hence could subsequently affect conclusions regarding nature of interactions between microbial extracellular polymers and their environment.

INTRODUCTION

Electron microscopy (EM)-based imaging and analyses provide excellent high-resolution tools for studying the structural and compositional features of microorganisms and their immediate surroundings. However, the conventional sample processing that includes dehydration as a pre-requisite for imaging by high-resolution vacuum instruments can cause substantial changes in microbial cell ultrastructure. Chemical
fixation with aldehydes and treatment with heavy metals help to preserve cell morphology and enhance contrast but can also result in substantial shrinkage (42). Dehydration with organic solvents can extract cell constituents, cause cell membrane discontinuities (23), induce distortion of delicate structures such as membrane-associated components (4), and have other deleterious effects to morphology that can consequently lead to inaccurate interpretations of cellular features.

To reduce the damage inherent to these treatments, various innovative cryogenic (cryo) sample preparation methods have been developed (14, 50). Introduction of the high-pressure freezing coupled with freeze-substitution process brought revolutionary improvements to the EM-based imaging of bacterial cells and associated extracellular material, allowing visualization of such sub-cellular structures as ribosomes and membrane components (25, 26, 60). In particular, cryo electron microscopy (cryoEM) enabled imaging of biological materials in near fully hydrated, closest-to-natural state (40). Based on preventing ice crystal growth by inducing rapid transformation of cellular water to amorphous ice (vitrification) during the sample preparation and imaging at sub-zero (°C) temperatures to lower the water vapor pressure under EM vacuum, cryoEM provided an exceptional technique for analysis of complex biological ultrastructures and macromolecular organization (20, 47). Devoid of the influences of chemical fixatives, dehydration, or heavy metal staining, cryoEM can provide morphologically accurate images of rapidly frozen living cells and various complexes (7, 54, 62). In fact, when thawed, the vitrified bacteria could regain more than 70% of viability (12).

Bacterially-secreted extracellular polymeric substances (EPS), forming the matrix of microbial biofilms, present a remarkable dynamic material that plays critical functional
roles in many important processes such as dental plaque formation, anaerobic corrosion of metals, attachment of pathogens to host cells, and a wide variety of geomicrobial processes in soil, sediment, rock, and marine environments (9, 61). EPS is primarily associated with physical adhesiveness, which plays a major role in bacterial flocculation, biofilm formation, cell adhesion to solid surfaces, and in creating protective microhabitats against adverse environmental conditions including desiccation, assault by anti-microbial agents, and oxidative stress (31, 41, 44). Additionally, due to its immense absorptive capacity, EPS is capable of binding, accumulating and sequestering dissolved organic matter and metals from the environment, and is consequently able to influence a wide range of biogeochemical processes. These processes include the dissolution and precipitation of minerals and the transformation of metals and radionuclides including redox and/or complexation reactions (10, 46).

Generally composed of bacterially-secreted heterogeneous combinations of high molecular weight polysaccharides, lipids, phosphate, proteins and nucleic acids, as well as of variable amounts of structural components such as flagella, pili, membrane vesicles, and cellular debris, EPS mass can consist of up to 95% of bound and unbound water (16, 53, 61). Due to this extreme hydration, EPS is among the most difficult biological structures to preserve and characterize in its native state, and presents a major challenge for obtaining accurate high-resolution images via electron microscopy.

Bacterial EPS has been described as “collapsing into the thick bundles” upon the exposure to traditional organic solvents during dehydration (2). Comparative reports on EPS visualization by EM (29, 52, 55) and studies focused on improving the EPS morphology by incubation with multivalent cationic dyes, heavy metals or gold-
conjugated lectins (13, 14, 35) greatly improved the morphological characterization of EPS. Unfortunately, these efforts still employed complete or partial dehydration steps which prohibited the visualization of EPS in its nearest-to-native, hydrated state. For obtaining precise structural information, approaches are needed that preserve the morphology of all bacterial components, especially extracellular material.

The scientific interest in microbial metal- and mineral interaction requires obtaining reliable information on the physical association of bacteria, EPS, and metals and/or mineral phases in the natural hydrated state. The selection of the cryoEM methods proved to be an effective tool for these investigations. The scheme of stabilizing the microbial material with vitreous ice, hence preventing its collapse in a vacuum during EM imaging, allowed the acquisition of accurate cellular morphological information that can be used for modeling bacterial processes; this also allowed documenting the unprecedented views of newly formed biominerals associated with the EPS. In addition, complementary EM techniques were used to directly observe dynamic process of water loss during dehydration, and to correlate these observations with fully hydrated cells and their surrounding EPS to obtain a more complete view of EPS collapse induced by water removal. Consequently, an analysis of the collected image data was provided in accordance with the concept of plastic deformation of viscoelastic material under stress conditions.

MATERIALS AND METHODS

Cells and media. (a) The washed, resting Shewanella oneidensis strain MR-1 (ATCC 700550^T) cell suspensions with a final density of 2x10^8 cells/mL were prepared as
previously described (37). The reduction assays were conducted in 30 mM sodium bicarbonate buffer (pH 7.0) with 10 mM sodium lactate as the sole electron donor and a final concentration of either (i) 250 µM U(VI) as uranyl acetate, (ii) 1.0 mM Mn(IV) as laboratory-synthesized mineral vernadite (32), or (iii) 1.0 mM sodium fumarate as the sole electron acceptors. The assay tubes were purged with N₂:CO₂ gas mixture (80:20, vol/vol) and sealed prior to the addition of cells, followed by horizontal incubation at 30°C with slow gyratory shaking (25 rpm). The reduction of U(VI) or Mn(IV) was determined as previously described (37, 38, 49). (b) Natural biofilms of sulfate-reducing bacterial consortia were obtained from a borehole used for long term (110-day) acetate injection during biostimulation activities at Department of Energy’s (DOE’s) Integrated Field Research Challenge site near Rifle, CO (http://ifcrifle.pnl.gov/). Small biofilm samples were removed from the exterior surface of tubing located within an injection borehole after 90 days of acetate addition. Samples were scraped from the tubing surface using a sterile razor blade, and the mineral-encrusted biofilms were immediately immersed and sealed in groundwater pumped from the sampling borehole before being shipped overnight at 4°C to the microscopy facility.

Cryo Transmission EM (cryoTEM) preparation. The Vitrobot freeze plunging apparatus (Mark III, FEI, Hillsboro, OR) was used for the cryo-immobilization of bacterial suspensions. Five µL of planktonic Shewanella cell suspensions were applied on freshly glow-discharged Quantifoil® R 2/2 grids (Electron Microscopy Sciences [EMS], Hatfield, PA). The cells were allowed to adhere to the grids for 30 seconds before blotting them twice (1s each, offset -1) on discs of filter paper placed in a Vitrobot humidified chamber to remove excess water. Cells in the remaining very thin aqueous
layer were immediately plunge frozen by immersion into a reservoir with liquid ethane cooled by liquid nitrogen within the Vitrobot instrument. The grids with frozen bacterial suspensions were transferred under liquid nitrogen to the Gatan 626 cryoTEM holder (Gatan Inc, Pleasanton, CA) using the cryo-transfer station (Gatan). After inserting the cryo-holder to the transmission electron microscope (TEM), the temperature was maintained below -178ºC at all times during the cryo-imaging unless noted.

**Correlated cryo- and room temperature- TEM.** During the cryoTEM imaging, the x and y coordinates of the bacterial features of interest were recorded using TEM compustage software. The cryo-holder was then removed from the TEM and transferred to the dry pumping station (DPS) (Gatan). The holder with the sample was gradually brought to room temperature (RT) using the warm-up cycle, reinserted into the TEM, and images of the specimen were collected while at RT. Although the marked positions did not align exactly with the material due to the general contraction of the sublimated material, it was easy to identify the previously imaged areas. The main advantage of this methodology was to eliminate rotational shift. To simplify the alignment of the images obtained under the two conditions, series of images were collected at fixed magnifications. The images were aligned using the “Layers” feature in Adobe Photoshop, and cell shrinkage in two dimensions (2D) was calculated by weighted correlation of 30 pairs of identical cells at both hydration conditions.

**Cryo Scanning EM (cryo SEM) and Focused Ion Beam (FIB).** (a) *S. oneidensis* MR-1 cells suspensions – 5µL drop was applied to a 200-mesh copper TEM grid with carbon-coated formvar support film (EMS) adhered by double-sided adhesive carbon tape to an aluminum stub on the cold stage holder. After 1 min, the excess liquid was removed
by wicking, and the sample was immediately plunged into the liquid nitrogen using a
device within the Quorum PPT2000 cryo-preparation stage (Polaron, Quorum
Technologies, UK). After the vacuum was applied to create a semi-slush consistency of
liquid nitrogen, the holder with the sample was raised and cryo-transferred at the
temperature of liquid nitrogen vapors to the cryo preparation chamber. To emphasize the
three dimensional (3D) cellular structures, the upper layer of amorphous water was
briefly sublimated at -95°C before the temperature was lowered to -160°C. The sample
was coated with Pt and transferred to the cooled stage of the Helios 600 nanolab Dual
Beam SEM (FEI) for imaging. (b) Biofilms: a small piece of fully hydrated natural
biofilm was transferred onto the cryoSEM cryo specimen holder coated with a carbon
tape, and allowed to adhere for 1 min. The residual liquid was removed with the tip of
filter paper and immediately plunged into the liquid nitrogen as in (a). The biofilm
sample was sputter-coated with a few nm of Pt in the cryo preparation chamber, and
transferred to the cooled stage of the Helios microscope. The samples were initially
imaged at -180°C by secondary electrons detectors; to emphasize the 3D structure of the
biofilm, the temperature was briefly raised (-160 °C for 2 min) to sublimate out (“freeze
etch”) the upper layer of amorphous water, including nanocrystalline ice on the very
surface. Furthermore, by using the heating stage, the temperature was increased with
20°C increments (in 10 continual steps from -160 to +20°C, allowing 20 minutes between
each temperature change). This way, the dynamic biofilm structural changes could be
observed during the dehydration. The images and real-time movies were acquired at each
time point for structural comparison of the cellular material (Movie S1). For the cryoFIB
of the selected biofilm regions, the stage was tilted to 52 degrees and a protective
platinum layer was applied before FIB milling. 30kV and beam current in range from 1-10 nA (10nA for rough milling, 1 nA for cleanup cut) in the area of 40 μm wide and approximately 15 μm deep was used to progressively cut and remove the material in the direction perpendicular to the biofilm surface.

**TEM preparation by high-pressure freezing (HPF) and automatic freeze substitution (AFS).** HPF/AFS, followed by plastic embedding was used to produce thin sections of (a) *S. oneidensis* MR-1 cell suspensions and (b) natural biofilms. (a) The cells were pelleted by brief centrifugation with a quick-spin minifuge, mixed with 0.5% agarose (Cambrex Bio Science Rockland, Inc. Rockland, ME), 3 μL of the suspension was transferred into a HPF flat specimen carrier, and frozen with a Leica EM PACT high-pressure freezer (Leica Microsystems Inc., Bannockburn, IL) at a typical rate of 1700ºC/s. The pods with compacted frozen cells were transferred under liquid nitrogen to the pre-cooled AFS (EM AFS, Leica), and a protocol for cell fixation, water substitution by acetone and a gradual warm up to RT was followed (Table S1). After 72 hrs, the samples were released from the pods by a gentle liquid flow induction in the surrounding acetone. The samples were washed 3x in acetone, gradually infiltrated with an ascending series of Spurr’s low viscosity embedding media (EMS) (25%, 50%, 75%, and 3x 100% washes, 120 min each), and cured at 60ºC for 24 hrs. The polymerized blocks were sectioned to 70 nm thin sections using Leica Ultracut UCT ultramicrotome, mounted on formvar-coated 100 mesh Cu TEM grids sputtered with carbon, and post-stained for 7 min with aqueous 2% uranyl acetate followed by 3 min Reynolds’ lead citrate (51) prior to TEM imaging. (b) The biofilms were infiltrated with 10% dextran for 15 min and prepared for HPF relative to their overall morphology. For thick biofilms, a
flat piece was dissected using a sample punch (Leica #706892) and the material was frozen in a specimen carrier using Leica EM PACT. For biofilms with more dispersed non-oriented structure, the material was manipulated into the copper specimen tubes of HPF pods using a cellulose microcapillary (Leica #706869) inserted into a Cu microtube to help the capillary action to drawing the biofilm into the tube. After the HPF, both types of biofilm samples were transferred under liquid nitrogen to the AFS unit and processed for plastic embedding as described above. No post-staining was applied due to the sufficient contrast provided by the soluble mineral portion.

**Scanning EM (SEM) preparation.** One mL of planktonic *S. oneidensis* MR-1 cells or a selected portion of bacterial biofilm was applied to the 0.2 µm polycarbonate track-etched membrane filters placed on top of filter paper in a Petri dish, and allowed cells to adhere for 1 minute. Excess liquid was removed by micro-puncturing the filter membrane with fine-tip forceps. The wet membranes were folded and transferred into vials where all subsequent washes were carried out to prevent cell loss during the numerous fluid exchanges. The samples were processed in one of three ways: (a) aldehyde fixation and ethanol dehydration followed by critical point drying (CPD); (b) ruthenium red - lysine fixation, dehydration, and CPD; and (c) air-drying. (a) The membranes with cells or biofilms were fixed in 2.5% glutaraldehyde (EMS) for 1 hr, washed 3 times in PBS and gradually dehydrated in an ethanol series (25%, 33%, 50%, 75%, 90% and 3 times 100%, 15 min each). After dehydration, the membranes with cells were placed into the pre-cooled processing chamber of a CPD instrument (Pelco CPD2, Ted Pella Inc., Redding, CA) and processed according to an automated CPD scheme, reaching a temperature of 35°C at 1200 psi, using CO₂ as a transitional fluid. The CPD-processed membranes were
mounted on standard aluminum SEM stubs covered with double-sided carbon adhesive
tape and sputter-coated with carbon. (b) To stabilize the delicate EPS structures, a
slightly modified ruthenium red–lysine fixation protocol (15) was followed. Briefly, the
samples were incubated with a stain cocktail containing 0.075% ruthenium red (Sigma,
St. Louis, MO), 30 mM lysine (Sigma), 2% paraformaldehyde (EMS), and 2.5%
glutaraldehyde in 0.1 M sodium cacodylate buffer (EMS). After 30 min, the membranes
were washed 3x in 0.1 M cacodylate buffer, dehydrated in ethanol series, and CPD was
performed as described above. (c) The air drying was carried out without ethanol
dehydration or CPD in selected samples to evaluate the effects of transitional solvents on
cell shrinkage. Samples were sputter-coated with carbon upon complete dehydration by
air drying. All samples were evaluated at RT in the SEM at 2kV.

**Microscopy.** TEM samples were examined using Tecnai T-12 TEM (FEI) operating
at 120 kV with LaB₆ filament, equipped with a high tilt stage for tomography and a
cryostage. Images were collected digitally using a 2×2K Ultrascan 1000 charge-coupled
device with a “U” scintillator (Gatan) calibrated to the TEM camera length to enable
direct measurements correlated with the magnification of the acquired images.
DigitalMicrograph™ (Gatan) software was used for imaging and analyses of cellular
features. CryoSEM imaging was performed at a Helios 600 Nanolab Dual Beam
microscope (FEI) coupled with a Quorum PPT2000 cryo-preparation stage (Polaron,
Quorum Technologies, UK). The room temperature high resolution imaging and the
electron energy loss spectroscopy (EELS) analysis (Supplemental Information) was
performed on a JEOL2010 TEM (JEOL Ltd., Japan) equipped with a Gatan Image Filter
GIF2000 (Gatan) and Gatan 1x1K CCD. Absorption edges determined by EELS were
analyzed by comparison of unknowns to standards of known oxidation state (19). For room temperature SEM, samples were evaluated in LEO 982 field emission SEM (Zeiss, Thornwood, NY).

RESULTS

CryoTEM. CryoTEM was used to acquire accurate ultrastructural image data on bacterial cells and associated EPS in their hydrated state. Bacterial suspensions prepared under controlled conditions were vitrified and imaged by cryoTEM at -178°C using the low-radiation-dose scheme (Fig. 1). The EPS produced by *S. oneidensis* MR-1 had a very low contrast due to the lack of electron density in this highly hydrated state and the absence of contrasting agents (Fig. 1B). The resulting cryoTEM images of the whole cells were used to obtain accurate measurements of cellular features such as the region between the outer surface of the plasma membrane and the inner face of the outer membrane (PM and OM), *i.e.*, the periplasm (24) (Fig. 1C). The dimensions of this region as determined by measurements from approximately 200 cells from each treatment were evaluated, and the mean width of the periplasm was determined to be 23.5±3.7 nm. This is approximately 25% thicker than values previously obtained by more traditional methods of thin sectioning of plastic-embedded cells processed by the high-pressure freezing and freeze substitution (Fig. 1A). Although measurements of the width of the periplasm in Gram-negative bacteria vary (57), the typical width in *Escherichia coli* is 12-15 nm (36). Since the frozen-hydrated bacteria are as close to the natural state as currently obtainable, these periplasmic distances are plausibly among the most accurate ones. In contrast with more traditional TEM preparations, other hydrated cellular
features, \textit{e.g.} the peptidoglycan layer (Fig. 1C) and ribosomes (not shown), were also identifiable. Vitrification also improved the visualization and spatial information of other cellular features such as blebbing of membrane vesicles (Fig. 1D).

**CryoEM of hydrated EPS and associated mineral phases.** Resting cell suspensions of the dissimilatory metal-reducing bacterium \textit{S. oneidensis} MR-1 were exposed to two metals in their most oxidized forms: (a) manganese (Mn(IV) and (b) uranium U(VI), respectively. These metals exhibit contrasting biogeochemical behavior as a function of oxidation state: nanocrystalline MnO$_2$, a poorly soluble solid under the given incubation conditions, is reduced under anaerobic conditions by \textit{S. oneidensis} MR-1 to Mn(II) which is relatively soluble (26, 45, 56). U(VI) is considerably more soluble, especially in the presence of complexing ligands such as carbonate relative to nanocrystalline UO$_2$ (uraninite), the primary product of U(VI) reduction (21, 34). The reaction kinetics for U(VI) and Mn(IV) reduction were characterized in our previous experiments, and the corresponding bacterial-mineral associations in the dehydrated state were imaged (18, 37). The high resolution images of bacterial cells and EPS in their hydrated state specifically associated with nanoparticulate Mn oxide and reduction end-product (\textit{i.e.} Mn(II) ions) were captured (Fig. 2). CryoTEM of cells incubated with nanocrystalline MnO$_2$ revealed that both the solid-phase Mn and soluble Mn are localized specifically within the EPS (Fig. 2A). The cryoSEM provided additional support for our cryoTEM observations as both cells and MnO$_2$ crystals were observed to be enrobed in an EPS matrix which became web-like and tapered into filaments upon the cryoSEM sample preparation (Fig. 2C). By contrast, RT TEM and SEM revealed that EPS shrank around MnO$_2$ crystals (Fig. 2B and D) and that collapsed EPS was untapered and appeared to
interconnect many cells after CPD preparation for RT SEM (Fig. 2D). For Mn species identification, the cryoTEM was coupled with the electron energy loss spectrometry (EELS) and high resolution TEM imaging at the RT mode (Suppl. Info and Fig. S1). The EELS showed mixed valence of Mn(II/III) associated with EPS produced by \textit{S. oneidensis} MR-1. The unprecedented views of specific associations of EPS with associated Mn(II) and Mn(III) ions further substantiates our knowledge of a significant role of EPS in microbial mineral and metal interactions.

\textbf{Changes in EPS morphology resulting from dehydration in vacuo.} To demonstrate the susceptibility of EPS to dehydration-mediated structural collapse, the fully hydrated EPS was subjected to relatively slow dehydration by sublimation induced \textit{in situ} under vacuum inside the TEM column, assisted by a slight temperature increase. These images captured how the originally highly hydrated, gel-like EPS undergoes dramatic physical changes (Figs. 3A-C). Upon exposure to a slow (~400s), controlled temperature rise from the initial -178°C to -155°C, the phase transformation from the amorphous ice started, including nanocrystalline ice nucleation, followed by the partial release of unbound water under vacuum as sublimation proceeded. The EPS revealed surprising pliability as it began to collapse and condense into fiber-like structures that increasingly stretched between the areas where the material was anchored to the grid support. This phenomenon is in a good accordance with documented behavior of viscoelastic material under stress conditions that induce plastic deformation (11).

\textbf{CryoTEM correlated with room temperature TEM.} To compare the cell architecture in both the hydrated and dehydrated states, a frozen-hydrated samples of \textit{S. oneidensis} MR-1 resting cells reducing uranyl, U(VI), to nanoparticulate uraninite
[U(IV)O₂⁺] were first observed in the cryoTEM (Fig. 3D), and subsequently brought to RT in a DPS to facilitate correlated imaging in dehydrated state. After reaching RT, the identical regions of the dehydrated sample that were previously imaged in the frozen-hydrated state were reimagined (Fig. 3E). The corresponding cells had undergone considerable collapse and shrinkage relative to their dimensions in the hydrated, vitrified state. Dehydration resulted in a decrease in average cell area of ~29 to 41% relative to the average cell area in images from vitrified samples (calculated from 2D projections, Fig. 3F). Moreover, the EPS underwent extensive condensation from the hydrated, gel-like state surrounding cells to a highly-collapsed state which was difficult to identify after dehydration. Notably, the nascent UO₂ nanoparticles and U(VI) ions that were specifically bound by the predominantly negatively charged, hydrated EPS provided (a) extra support to the EPS matrix (nanocrystalline mineral phase), and (b) additional contrast due to the higher electron density, essentially acting like a post-stain.

**CryoSEM and FIB examination of biofilms.** In addition to the controlled laboratory studies of axenic *Shewanella* cultures, the morphology of mature, natural biofilms obtained from a uranium bioremediation field research site near Rifle, CO (1, 59) was also investigated. In the hydrated state at -180°C, the biofilm surface was covered by a smooth, thin layer of gel-like EPS enrobing bacteria (Fig. 4A, B). Upon sublimation to -150°C, cells became more pronounced within the EPS matrix, and the first signs of the EPS collapse could be observed, becoming more pronounced around -90°C (Fig. 4C, D). Analogous to the cryoTEM observations of *S. oneidensis* MR-1 EPS, the dynamic structural changes of biofilms resulting from gradual dehydration in the cryoSEM were documented by real-time imaging (Movie S1). Sublimation induced distortion of biofilm
surface features as extensive cracks formed in the initially cohesive material over time (Fig 5A and B). The EPS structural integrity loss became exacerbated by the overall material shrinkage, creating an increased strain on the viscoelastic matrix. As the mechanical stress during sublimation progressed or after CPD preparation of samples, matrix collapse was observed first in the form of branched, web-like strands, followed by the formation of rope-like filamentous structures often greater than 10 μm in length (Fig. 5A-D). The layer beneath the biofilm surface was imaged by creating a cross-section by in situ FIB milling of biofilm maintained under cryo or RT conditions. The cryoFIB-generated cross-section revealed bacteria with different degrees of associated mineralization, as well as secondary mineral phases devoid of bacteria (Fig. 5 E and F).

Images of EPS and bacteria in biofilms prepared by additional EM processing methods are included in the Supplemental Information for comparative purposes (Figs. S2 and S3).

DISCUSSION

The highly hydrated associations of bacteria and EPS interacting with minerals and metals were systematically investigated using an array of cryo and traditional EM techniques. The resulting conceptual model and respective correlated images illustrating the EPS structural changes induced by various EM sample processing methods is presented in Fig. 6. Clearly, cryoTEM is a superior method for maintaining the cells and EPS in their highly hydrated state with excellent ultrastructural preservation (Fig. 6A, B). This methodology also produced extremely accurate measurements of the region exterior to the plasma membrane; the location of the catalytic electron transfer proteins in S.
*oneidensis* MR-1 essential for metal and radionuclide reduction (3, 43). The amorphous structure of the EPS was observed; however, due to the high water content and light element composition, EPS has a very low electron density, resulting in low contrast (Fig. 1B). The visualization of this material by cryoTEM was enhanced when soluble electron-dense metals [U(VI) or Mn(II)/(III)] were present (Figs. 2 and 3). RT TEM of frozen-hydrated cell suspensions subsequently sublimated in a weak vacuum of the DPS resulted in EPS dehydration and collapse into mesh-like structures that maintained the overall “footprint” of the hydrated EPS (Fig. 6C, D). Interestingly, the samples prepared by DPS sublimation also demonstrated improved spatial preservation of overall 3D structure even after progression to full dehydration. This was in contrast to the complete z-direction collapse of cellular and EPS material observed with air-dried samples (TEM whole mounts – Fig. 6E, F). When negatively stained with an electron-dense solution, bacterial appendages such as flagella and pili can be imaged in great detail by this technique (Fig. 6F). CryoSEM maintained cells and EPS in a highly hydrated state, with the exception of a thin surface water layer that was intentionally sublimated to mitigate the build-up of water ice (Fig. 6G, H). If the temperature was further increased, thinning of the EPS due to the water loss became more prominent, and signs of dehydration such as the loss of the EPS integrity started to appear. The deformation of viscoelastic EPS material that was observed during sublimation resulted in the creation of features resembling the filamentous structures commonly present in dehydrated preparations (Movie S1). In sample preparation by CPD, the solvents such as ethanol or acetone used as a transitional fluid for replacing cellular water can initially extract lipids and protein complexes, resulting in changes in cellular dimensions (shrinkage) (Fig. 6I, J). During the CPD
process, after the substitution of transitional fluid with liquid CO₂, the material is exposed to simultaneous stresses of a strong laminar flow in the closed microfluidics environment, to a very high pressure and increased temperature inducing the phase transformation, and finally to complete dehydration. The exceptional preservation of turgid cells is a signature of this method; however we speculate that loosely supported EPS undergoes notable transformation, resulting in the distinctive filamentous connections between the cells and minerals. The structures would be predicted, based on the behavior of viscoelastic material, to have a narrowing region at the base of their attachment. However, these filaments, without the pronounced tapering region, are observed specifically in the CPD preparations. We believe these are secondary products of chemical processing and viscoelastic deformation of the EPS induced by this method.

There are two unsettled questions regarding bacterial EPS structure and function: First, **What happens to this complex network** of carbohydrate macromolecules, proteins and lipids that entraps organic matter and contains up to 95% water and, in many cases, binds metal ions, **when water is removed?** The EPS can be expected to display rheological behavior of complex fluid under these conditions (33). During the dehydration process, functional groups are deprived of their –OH group, and as molecules of water are removed, condensation reactions on the EPS components occur. The originally flexible polysaccharides become rigid as they lose their glycosidic bonds that provide them with conformational flexibility. Proteins, partially denatured in the fixation step by aldehydes that cause crosslinking, are dehydrated by organic solvents such as ethanol or acetone, further inducing communal aggregation (6). In addition, organic solvents partially remove lipids, contributing to microstructure breakdown. It is
also likely that shear conditions during the sample preparation may induce assembly of structures in a more dense form around linear polysaccharides (“backbone”), becoming increasingly rigid as the dehydration progresses. Together, these conditions promote the sol-gel phase transition of EPS to a network of interconnected fibrils and densification. Finally, upon further water removal, the viscosity of this supramolecular hydrogel increases, and eventually transformation to a solid state occurs.

The second question is: **What happens to ions in EPS upon dehydration?** During the exposure to cationic metals, either naturally in solution or resulting from microbial biotransformation, hydrated EPS will bind the ions, concentrating them within the matrix (5). When EPS loses hydroxyl groups during dehydration, the increasing concentration of the ions within the contracting polymer chains in the confined space will effectively increase the ionic strength that can result in the precipitation of salts, with implications on creating microenvironments with different physical and chemical properties.

Bacterial EPS can influence important geochemical processes including oxidation/reduction, dissolution/precipitation, colloid transport, subsurface water flow, and contaminant movement in situ (17). Although the exact role(s) of EPS produced by *S. oneidensis* MR-1 during metal reduction remain undetermined, its production upon exposure to soluble or solid electron acceptors and the extracellular occurrence of reduced metals suggests that EPS could be an important component of this organism’s extended electron transport system. Some metal-reducing *Shewanella* strains have been reported to secrete flavin-based electron shuttles (39, 58), produce EPS that contains outer membrane c-type cytochromes (37, 49), and evolve electron-conducting nanowires (22) under conditions of electron acceptor limitation, indicating that this organism may
use multiple mechanisms for extracellular electron transfer. Whether hydrated EPS has a direct role in extracellular electron transfer or an indirect one, such as providing a supporting matrix for redox proteins, low molecular electron shuttles, or nanowires, remains to be determined.

Filamentous EPS structures observed in bacterial samples prepared and imaged by traditional EM methods have been suggested to be involved in, directly or indirectly, physical interactions and aggregation (28, 48), cell-to-surface attachment by tethering via thin adhesion threads (30), extracellular electron transfer (22), and possibly *de facto* electrical signaling within the structurally integrated communities (8). Although these are intriguing observations, our analysis illustrates how dehydration can severely alter chemical and physical properties of bacterial EPS that in turn can influence mechanistic interpretations of microbial processes. It is critical that the potential for generation of dehydration-induced changes on EPS structure and function is recognized and, when possible, avoided or minimized through the use of advanced cryoEM methodologies that will enable further advances in understanding bacterial interactions with their environment.

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We dedicate this manuscript to our colleague and mentor, a world-renowned geomicrobiologist Prof. Terry J Beveridge (†2007) whom we regard as the true pioneer in the high-resolution characterization of structural microbiology, with his remarkable contribution in areas such as bacterial cell wall reactivity and microbial effects on metal speciation.

REFERENCES


**FIGURE LEGENDS**

**FIG. 1.** Comparison of fine-scale bacterial cell features as revealed by HPF/AFS processing and cryoTEM. The morphology of *S. oneidensis* MR-1 cells processed by HPF followed by AFS (A) showed the most accurate cell features of all traditional chemical fixation sample preparation methods including intact periplasm; with some separation in the membranes area (A - arrows). EPS appears condensed into random structures that are difficult to interpret in a cross-section (asterisks). (B) Bacteria imaged by cryoTEM display exceptionally well pronounced membranes; however the EPS has a very low contrast, due to the high water content and generally by the relatively dispersed nature of light elements. (C) Distinct features, such as outer and plasma membranes (OM and PM, respectively) defining the periplasm (P) and the peptidoglycan layer (PG) provide accurate morphological information that can be used for obtaining precise dimensions. CryoTEM also provides improved ultrastructural detail in bacterial processes, *e.g.* (D) a membrane vesicle pinching off of a cell. Resting cell suspensions of *S. oneidensis* MR-1 were incubated anaerobically with lactate and fumarate and
representative assay aliquots were prepared for concurrent EM imaging. Scale bars are 200 nm (A,B,D) and 500 nm (C).

**FIG. 2.** Comparison of bacteria and EPS in the hydrated and dehydrated state. Resting cell suspensions of *S. oneidensis* MR-1 were incubated anaerobically for 24 hours with lactate and MnO₂ and representative assay aliquots were prepared for concurrent cryo and RT EM imaging. (A) CryoTEM micrograph of cell illustrating EPS in the hydrated state. Slight contrast of the EPS is likely due to bound Mn(II) resulting from dissimilatory reduction of Mn(IV). Arrow in (A) shows the MnO₂ crystalline material. (B) The whole mount RT TEM preparation shows a cell with the collapsed EPS covering the MnO₂ material (arrow). (C) Representative cryoSEM image of rapidly frozen material after partial sublimation displays partially hydrated cells associated with EPS that covers the MnO₂ material (asterisks). (D) Fixed, dehydrated, and CPD-prepared sample characteristically shows excellent 3D cellular preservation, but completely altered EPS that forms fiber-like structures co-localized with MnO₂ material (asterisks). Scale bars are 500 nm.

**FIG. 3.** (A-C) Progressive transformation of *S. oneidensis* MR-1 EPS during dehydration in the cryoTEM. (A) Partly hydrated EPS of washed resting cells incubated with lactate and fumarate and without potential contrasting metal ions, supported by a carbon film (dashed circle outlines a hole in the support film). (B,C) An assortment of secondary-formed nanocrystalline ice particles covers the EPS surface. Large hexagonal ice crystals (asterisks) can serve as fiducial markers during the dynamic progression of the EPS
dehydration. Arrows point to the newly formed EPS structures that form during transformation of the viscoelastic EPS that collapses due to the water loss during sublimation and non-elastic stretching between the points anchored to the carbon film. Time sequence from A-C was 200 seconds, with electron beam blocked between the three low dose exposures at temperature -152ºC. (D-F) Comparison of cells and associated EPS surface area in hydrated state and after subsequent dehydration. (D) CryoTEM image (-178 ºC) of cells incubated for 24 hr with U(VI) and lactate. The visualization of EPS is enhanced by contrast from soluble U(VI), and nascent UO₂ nanoparticles are observed within hydrated EPS. (E) Identical area after DPS sublimation to RT. (F) Superimposed cryoTEM image with the outlines of the same two cells over the RT image illustrating a substantial shrinkage and detachment of the EPS with the UO₂ precipitates distal to the cell surface (arrow). Estimated cellular linear volume loss was 29-41%. Scale bars in (A-F) represent 1µm.

FIG. 4. Process of gradual moisture loss from frozen-hydrated natural biofilm as captured by cryoSEM. (A,B) Initial image obtained at -180ºC in fully hydrated state after the biofilm was plunge-frozen. Black arrow in (B) points to a contour of bacteria embedded in EPS material that remains partially hydrated. (C,D) The same area of biofilm after sequential warming to -150ºC. Notice the substantial collapse in the z-direction (thickness), and beginning of the shrinkage in the x, y dimensions resulting in cracks (asterisk in C). White arrows in B and D highlight the structural alteration of EPS, with the formation of a filamentous structure. The cellular features in D (black arrows) are more pronounced after sublimating a thin layer of the water from the surface. Black
FIG. 5. Observation of biofilm features by cryo and RT SEM. (A,B) EPS transformation during sublimation induced by progressive warming in the cryoSEM. Biofilm shows substantial structural damage due to material collapse and contraction induced by warming to -60°C. (A) Distortion of biofilm surface features with extensive cracks (>200 μm) developed over time in the initially cohesive material. (B) Formation of filamentous structures could be observed in situ during the surrounding material shrinkage while the partially hydrated viscoelastic EPS material was stretched between apparent anchored points. (C,D) Morphologically similar filamentous structures formed during sublimation were also observed in samples prepared by CPD and viewed at RT in the SEM. (E) CryoFIB milling of an approximately 20 μm depth below the biofilm surface revealed superior structure preservation. (F) CryoFIB-prepared area with minerals-laden layers of EPS and cells in a various degree of mineralization (asterisks), with no signs of EPS collapse and stretching. Black boxes in C and E illustrate the areas shown in higher magnification in D and F, respectively. Scale bars: (A) 200 μm, (B) 5 μm, (C) 2 μm, (D) 500 nm, (E) 30 μm and (F) 2 μm.

FIG. 6. Model and correlated EM images of cells and associated EPS structural alteration resulting from different methods of sample processing. (A,B) CryoTEM of frozen-hydrated cells vitrified in amorphous ice imaged at -178°C. Arrows indicate the outline of the EPS. (C,D) RT TEM of initially vitrified cells that have been gently dried
in low vacuum of the DPS. (E,F) RT TEM of air dried cells, stained with Nano-W
(Nanoprobes, Yaphank, NY) prior to imaging. (G,H) CryoSEM of cells in a thin layer of
partially sublimated amorphous ice. (I,J) RT SEM of cells that were fixed, dehydrated,
and CPD prepared. (C), (E), and (I) also show hydrated cell outline for comparison of cell
shrinkage.
FIG. 1, Dohnalkova et al.
FIG. 2, Dohnalkova et. al.
FIG. 3, Dohnalkova et. al.
FIG. 4, Dohnalkova et. al.
FIG. 5, Dohnalkova et. al.
FIG. 6, Dohnalkova et. al.
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