

Visualization of Hydrated Bacterial Structures by Complementary Electron Microscopy Techniques

Matthew J. Marshall¹, Alice C. Dohnalkova¹, Bruce W. Arey¹, Kenneth H. Williams² and James K. Fredrickson¹
¹Pacific Northwest National Laboratory, Richland, WA 99354; ²Lawrence Berkeley National Laboratory, Berkeley, CA 94720



Introduction and Objective

A range of anaerobic and facultative microorganisms can biotransform metal and radionuclide contaminants of concern at DOE sites. Under conditions of metal reduction, many of these organisms produce extracellular polymeric substances (EPS) that are in association with newly formed nanoparticles of reduced uranium (i.e., uraninite, U(IV)O₂).

EPS are a highly hydrated network of lipids, polysaccharides, and protein complexes. These highly hydrated materials are susceptible to collapse during dehydration associated with conventional EM sample preparation.

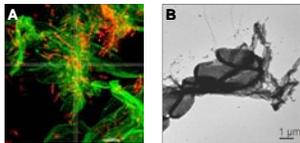
Cryo Electron Microscopy (cryo EM) imaging and analyses have potential to provide a more accurate image of the associations between native, hydrated bacteria, EPS, and metal ions/biominerals.

Cryo EM experiments with *Shewanella* cells incubated anaerobically with MnO₂ particles or soluble U(VI) suggest that our current knowledge of the interaction between EPS and minerals is strongly influenced by dehydration artifacts.

In this joint SFA-EMSL activity, we used cryo EM to image, under closest-to-native environmental conditions, the associations between bacterial cells, EPS and mineral precipitates. To accomplish this goal, we focused on the following objectives:

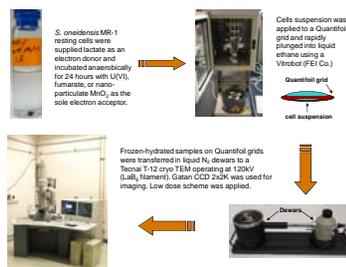
- Develop sample preparation methods for cryo Transmission EM (TEM) and cryo Scanning EM (SEM) to preserve bacteria and bacterial EPS in their closest-to-native state.
- Use an interrelated approach of both cryo- and traditional- TEM/SEM to elucidate the high-resolution interactions of bacterial EPS with metals, radionuclides, and bacterial cells.
- Demonstrate the use of cryo EM as an enhanced visualization method for fully hydrated, fine-scale interactions within natural biofilms produced during *in situ* biostimulation.

Previous studies

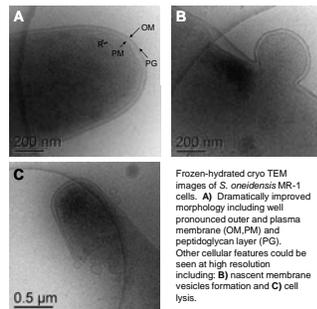


Our previous work demonstrated that it was difficult to visualize fully-hydrated EPS at high resolution. A) Confocal Laser Scanning Microscopy (CLSM) showing cells (stained red) in an extended matrix of EPS (stained green). B) Conventional TEM showing cells entangled in collapsed UO₂-EPS after drying. CLSM image by taken Jeff McLean (now located at the J.C. Venter Institute).

Sample preparation for cryo TEM

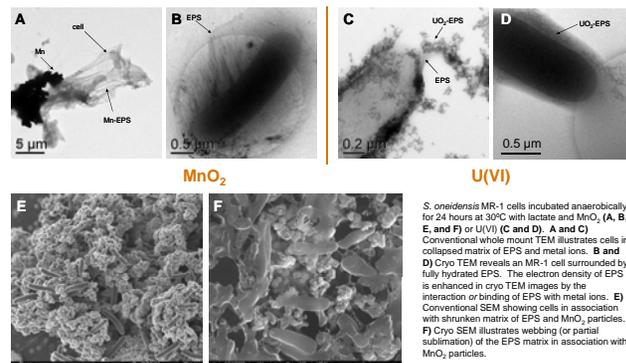


Cryo TEM produced dramatically improved cell morphology



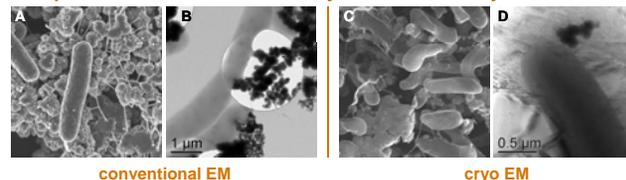
Frozen-hydrated cryo TEM images of *S. oneidensis* MR-1 cells. A) Dramatically improved morphology including well pronounced outer and plasma membrane (OM, PM) and peptidoglycan layer (PG). Other cellular features could be seen at high resolution including: B) nascent membrane vesicles formation and C) cell lysis.

Enhanced visualization of fully hydrated EPS interacting with minerals



S. oneidensis MR-1 cells incubated anaerobically for 24 hours at 30°C with lactate and MnO₂ (A, B, E, and F) or U(VI) (C and D). A and C) Conventional whole mount TEM illustrates cells in collapsed matrix of EPS and metal ions. B and D) Cryo TEM reveals an MR-1 cell surrounded by fully hydrated EPS. The electron density of EPS is enhanced in cryo TEM images by the interaction or binding of EPS with metal ions. E) Conventional SEM showing cells in association with shrunken matrix of EPS and MnO₂ particles. F) Cryo SEM illustrates webbing (or partial sublimation) of the EPS matrix in association with MnO₂ particles.

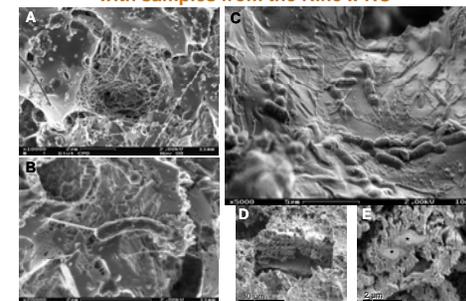
Comparison of conventional and cryo EM reveals dehydration artifacts



To further illustrate how hydrated EPS may undergo morphological changes during EM sample preparation and microscopy, a comparison of four different EM preparations was performed on a single *S. oneidensis* MR-1 culture incubated anaerobically with lactate and MnO₂. A) Conventional SEM using a series of glutaraldehyde fixation and ethanol dehydration steps followed by critical point drying and visualization at room temperature. B) Conventional TEM after sublimation and visualization at room temperature. C) Cryo SEM after freezing in liquid N₂ and partial sublimation at -95°C prior to cryo imaging. D) Cryo TEM of frozen hydrated cell after vitrification in liquid ethane and low dose cryo visualization.

Images demonstrate that cryo preparation and visualization prevents massive collapse of EPS and provides an image of near-native conditions!

Enhanced visualization of EPS associated with samples from the Rifle IFRC



To demonstrate the ability of cryo EM to visualize EPS associated with natural biofilms, a mature biofilm produced during *in situ* biostimulation activities at the DOE's Rifle, CO Integrated Field Research Challenge (IFRC) site was subjected to either conventional SEM or cryo SEM preparation. A and B) Conventional SEM images showing glutaraldehyde fixed, alcohol dehydrated, and critical-point dried biofilms. Cells are visible in constricted EPS. C) Cryo SEM of an identical biofilm sample. Multiple cell morphologies are visible in a concentric veil of EPS. D and E) Cryo Focused Ion Beam (Cryo FIB) and cryo SEM were used to visualize materials beneath the surface of the biofilm. The asterisk (*) in E indicates sectioned cells within the biofilm matrix.

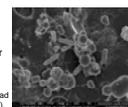
Summary and Conclusions

Cryo EM presents a new dimension in the imaging of microbial interactions with minerals and metals. Vitrification and low-dose imaging provide a dramatic improvement of cell architecture preservation and the visualization of hydrated EPS. Cryo EM imaging indicates that dehydration artifacts are often observed when imaging EPS of specimens prepared using conventional EM techniques. Current studies suggest that both cryo TEM and cryo SEM will significantly improve our understanding of bacterial-mineral associations *in situ*.

Future Research

In conjunction with the Hanford IFRC, we illustrate that a complex microbial community colonizes BioSep beads (provided by Lin & Konopa) deployed as part of an in-well experiment in the Hanford 300A. Cryo EM will permit us to visualize microenvironments inhabited by EPS-producing microbial communities at the Hanford IFRC site. Understanding the hydrated structure of these communities will provide vital information towards predicting their influence on subsurface pore-scale transport. Our perception of microenvironments where biogeochemical electron transfer redox reactions occur will be greatly enhanced by the use of cryo EM technologies.

Conventional SEM image of BioSep bead from well 300-9-24 (Hanford IFRC site).



Research performed through PNNL's Scientific Focus Area (SFA)
 Supported by DOE Office of Science
 Office of Biological and Environmental Research (BER)
 Climate and Environmental Sciences Division (CESD)



Electron microscopy was performed at the William R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the U.S. Department of Energy's (DOE) Office of Biological and Environmental Research (OBER) and located at PNNL. Financial support was provided through an EMSL Research and Capability Development Proposal and the work was performed as a joint-SFA-EMSL activity. PNNL is operated for the DOE by Battelle Memorial Institute under Contract DE-AC05-76RLO 1830.

www.pnl.gov