Introduction

This exploratory project involved laboratory experiments to investigate three hypotheses: (H1) Physics-based modeling of low-frequency dispersions (henceforth referred to as alpha) measured in broadband dielectric spectroscopy (DS) data can quantify pore-scale geometric changes impacting contaminant transport resulting from biomineralization; (H2) Physics-based modeling of high-frequency dispersions (henceforth referred to as beta) measured in broadband dielectric spectroscopy data can quantify rates of mineral growth in/on the cell wall; (H3) Application of this measurement and modeling approach can enhance geophysical interpretation of bioremediation experiments conducted at the RIFLE IFC by providing constraints on bioremediation efficiency (biomass concentration, mineral uptake within the cell wall, bio-mineralization rate). We tested H1 by performing DS measurements (alpha and beta range) on iron (Fe) particles of dimensions similar to microbial cells, dispersed within agar gels over a range of Fe concentrations. We have tested the ability of the physics-based modeling to predict volume concentrations of the Fe particles by assuming that the Fe particles are polarizable inclusions within an otherwise nonpolarizable medium. We evaluated the smallest volume concentration that can be detected with the DS method. Similar experiments and modeling have been performed on the sulfate-reducing bacteria D. vulgaris. Synchrotron x-ray absorption measurements were conducted to determine the local structure of biominerals coatings on D. vulgaris which were grown in the presence of different Fe concentrations. We imaged the mineral growth on cell wall using SEM. We used dielectric spectroscopy to differentiate between iron sulfide precipitates of biotic and abiotic nature. Biotic measurements were made on *D. vulgaris* bacteria grown in the presence of different concentrations of iron to form different thicknesses of iron sulfide precipitates around themselves and abiotic measurements were made on different concentrations of pyrrhotite particles suspended in agar. Results show a decrease in dielectric permittivity as a function of frequency for biotic minerals and an opposite trend is observed for abiotic minerals. Our results suggest that dielectric spectroscopy offers a non-invasive and fast approach for distinguishing between abiotic and biotic mineral precipitates.

Experimental description

Dielectric spectroscopy (DS) measurements were performed using a 2-electrode set up commonly used in the field of Biophysics.

Our first experiment was designed to test the ability of a physics-based theoretical model to predict volumetric concentrations of *Desulfovibrio vulgaris* in suspension. Whereas this theoretical model has been previously used to model spherical cells, it requires modification to deal with the distinct tubular shape of *Desulfovibrio vulgaris*. This experiment involved making measurements of *Desulfovibrio vulgaris* cells suspensions at a range of cell concentrations.

Our second experiment was designed to test the ability of a physics-based theoretical model to predict volumetric concentration of metallic targets representing biominerals. We started with an abiotic analogue where we used metallic minerals (firstly pyrrhotite) in suspension, and subsequently in porous media, to simulate biominerals. We homogeneously dispersed a known volume of metallic targets in suspension (gel) and perform DS measurements. We processed the DS data and estimate the volume of the targets based only on the physics-based modeling. We

proceeded with experiments on dispersed biominerals where we used the physics-based modeling to predict the change of the biomineral coating thickness.

Work conducted

Measurement and modeling of the dielectric responses of biotic versus abiotic iron sulfide using dielectric spectroscopy

D. vulgaris are sulfate-reducing bacteria that play an important role in the bioremediation of heavy metals and radionuclides. In the process of reducing and immobilizing these contaminants, they convert soluble heavy metal ions in water to insoluble metal sulfides, coating themselves with these nano-scale precipitates. To help evaluate real time bioremediation processes, distinction between already present abiotic mineral precipitates and actively forming biominerals is important. We used dielectric spectroscopy, a non-invasive and rapid technique to differentiate between iron sulfide precipitates of biotic and abiotic nature. Biotic measurements were made on *D. vulgaris* bacteria grown in the presence of different concentrations of iron to form different thicknesses of iron sulfide precipitates around themselves and abiotic measurements were made on different concentrations of pyrrhotite particles suspended in agar. Results show a decrease in dielectric permittivity as a function of frequency for biotic minerals and an opposite trend is observed for abiotic minerals. Our results suggest that dielectric spectroscopy offers a non-invasive and fast approach for distinguishing between abiotic and biotic mineral precipitates.

We investigated and compared the dielectric properties of biotic and abiotic iron sulfide minerals to see if there are diagnostic dielectric signatures associated with the two systems. These differences could help monitor the status of a bioremediation process by estimating the precipitate on the microbial wall and amount of abiotic precipitates that are not active anymore. We report a distinct difference in dielectric trend for different concentrations of biotic and abiotic iron sulfide minerals. Moreover, we propose that by differentiating between old/immobile (abiotic) precipitates and new/currently forming (biotic) ones, we can quantify the early phases of biomineralization using DS.

We measured the dielectric parameters of 1) *D. vulgaris* cells grown in the presence of different concentrations of iron in the growth medium as well as, 2) different concentrations of abiotic pyrrhotite particles that are of comparable size to *D. vulgaris* bacteria. We also carried out scanning electron microscopy (SEM) and X-Ray absorption near edge structure (XANES) spectroscopy on these cells to confirm biomineralization.

Methods:

Abiotic System. Abiotic measurements were made on pyrrhotite (Ward's Natural Science) particles with diameter less than 38 μm. Particles were mechanically crushed (Braun Chipmunk Jaw Crusher), sieved and suspended in 1% agar gel (Acros Organics). The density of these particles is high and it was difficult to obtain a uniform suspension in water. Hence these particles were suspended in 1% agar gel to obtain a well-dispersed mineral phase. Different concentrations of metal particles were measured, including 0.0%, 0.1%, 0.3%, 0.5%, and 0.7% by weight of metal. For 0.1% pyrrhotite, 0.015 g metal was added in a 15 ml centrifuge tube. Hot Agar gel was poured into the tube and the contents were mixed using a vortex and stirred vigorously, encouraging the metal sample to stay in suspension in the gel. The mixture was then poured on the bottom capacitor of the experimental setup and allowed to cool. For 0.3%, 0.045 g metal was added to agar mixture, 0.5% metal, 0.075 g metal was added to agar mixture, for 0.7% metal, 0.105 g metal was added and the same procedure was repeated.

Biotic System. *D. vulgaris* bacteria (ATCC 29579) were obtained from American Type Culture Collection. These cells have been most commonly used in bioremediation experiments and hence were used in our experiments. Cells were grown in modified lactate sulfate medium (LS4D). *D. vulgaris* (10% by volume) were inoculated in LS4D in an anaerobic chamber and incubated at 30˚C till they reached early stationary phase (48 hours). Cells were also grown in LS4D + 0.025 g/l Fe, LS4D+ 0.05 g/l Fe and LS4D + 0.15 g/l Fe to determine the effect of different concentrations of iron precipitation on cells. Additional iron was added in the form of $FeCl_2.4H_2O$ (Fisher). All the cells were harvested at 48 hours for easy comparison between cells grown in the presence of different iron concentrations.

Dielectric Spectroscopy. DS measures the dielectric permittivity (ε) and conductivity (σ) of samples as a function of frequency. All DS measurements were carried out using a Solartron 1260 impedance analyzer. Samples were placed between two parallel-plate electrodes made of gold with a 56.4 mm diameter. The distance between the electrodes was 3mm. The applied voltage was 0.15 V, below the linear threshold of 1 V/cm (see supporting information). Abiotic and biotic measurements were carried out from 1 Hz to 1 MHz. For biotic measurements, cells were incubated for 48 hours then centrifuged at 1500 x g for 5 min; the pellet was resuspended in 5 mM HEPES (Fisher) at pH 7. The cells were centrifuged again at 1500 x g for 5 min to wash the excess iron in the solution. The cells were then resuspended in 5 mM HEPES to OD_{600} of 0.1, as is commonly used for cells. The cell suspension was then placed between the capacitor plates for measurements. DS measurements were carried out under aerobic conditions. Each measurement lasted less than 10 min, ensuring viability of cells during measurements. All measurements were repeated six times on different samples.

Theoretical Model. A theoretical model was used to analytically solve for the dielectric response of live cell suspensions when subjected to an electric field. This model provides an analytical solution for spherical cells. Analytical solutions for cylindrical cells are more complex and involve several parameters that are not well constrained by our experiments. Hence, the spherical cell model was adopted here for simplicity. A cell is represented as a conducting sphere covered with an insulating thin shell representing the membrane. The membrane, the inside and outside of cells are each characterized by their own set of dielectric parameters. The dielectric response of a suspension of such cells is analytically calculated.

X-Ray Absorption Near Edge Structure (XANES). *D. Vulgaris* cells were measured using XANES spectroscopy to detect the presence of iron sulfide in the cells. *D. vulgaris* cells were cultured in LS4D medium, inoculated for 48 hours then centrifuged at 4000 RPM for 30 min. The tubes were transferred to the anaerobic chamber and the supernatant was discarded. The cells were then washed with 5 mM HEPES, centrifuged again at 4000 RPM for 30 min and transferred back to the anaerobic chamber. The supernatant was discarded again and cells were placed in the sample holders made of kapton sheets coated with aluminum. The sample holders were vacuum-sealed to avoid contact with oxygen. XANES measurements were conducted at the National Synchrotron Light Source beamline X19A, at Brookhaven National Laboratory. Spectra for the sample were collected in fluorescence mode using a Lytle type detector (ion chamber with Ar gas). The raw data for the FeS_2 Pyrite standard were obtained from the XAFS Data Library.

Scanning Electron Microscopy (SEM). SEM analysis was performed to determine the presence of precipitates on cells. *D. vulgaris* cells were grown in LS4D medium in the presence of 0.0 g/l, 0.025 g/l and 0.05 g/l Fe^{2+} for 48 hours. The cells were centrifuged at 1500 x g for 5 min, transferred to anaerobic chamber and the supernatant was discarded. Cells were washed with 5 mM HEPES to remove excess growth medium from the sample and centrifuged again at 1500 x g for 5 min. The sample was again transferred to an anaerobic chamber and supernatant was discarded. Silicon wafer was used as the substrate for SEM analysis. The cell pellet was transferred to the silicon wafer. The sample was fixed in 2.5% gluteraldehyde in phosphate buffer for 4 hours. The sample was then immersed in phosphate buffer for an hour, washed in a series of ethanol-water mixtures (10%, 25%, 50%, 75%, 90%) and finally rinsed three times in 100% ethanol to remove all the water from the sample. The sample was then air-dried overnight and SEM analysis was carried out.

Results

Abiotic Measurements. We measured the relative dielectric permittivity of different concentrations of pyrrhotite particles, ranging from 0% pyrrhotite (agar only) to 0.7% pyrrhotite in agar as a function of frequency. The data indicate that at low frequency, the dielectric permittivity of pyrrhotite particles increases with an increase in pyrrhotite concentration. The dielectric permittivity of a sample increases with an increase in its volume fraction according to the Lorentz formula. These results are also in line with previous studies that have shown an increase in the imaginary conductivity of the samples with an increase in their concentration. 0.05% and 0.01% pyrrhotite particles were also measured but the changes in dielectric permittivity with respect to 0% pyrrhotite were below sensitivity. Thus, 0.1% pyrrhotite was found to be the lowest concentration of pyrrhotite in a sample that could be detected using our experimental set up.

Biotic measurements. We measured the variation of dielectric permittivity of *D. vulgaris* cells at frequencies ranging from 1 Hz to 1 MHz. The cells were washed in 5 mM HEPES to remove excess iron from the solution before measurements. Data show that, opposite to the abiotic pyrrhotite, the relative dielectric permittivity of the cells decreases with an increase in the concentration of iron in the solution.

Phase values were calculated at 50 Hz for pyrrhotite particles and cells and plotted as a function of iron concentration. From the plots, we see that the relative dielectric permittivity, conductivity and phase of the pyrrhotite samples increase with an increase in their concentration. This trend is opposite to that observed for the cells.

We compared the Fe K-edge x-ray absorption near edge structure for the cell sample with a FeS_2 pyrite structure. The primary difference that can be seen is broadening of the features in the cells compared to the FeS_2 standard. This broadening is associated with loss of structural coherence and loss of long-range structure typical of the formation of an amorphous phase or the formation of a nanostructured material (with large number of atoms at the surface compared to the bulk). We further looked at the atomic coordination by taking the Fourier transform of the high energy or fine structure (structure factor) to probe the atomic shells about Fe. We compared the structure factor for the cells and standard. Both spectra reveal a dominant peak corresponding to Fe-S first neighbor shell. Fits to the sample data show that this Fe-S peak occurs at a distance of 2.305 ± 0.013 Å for the cells and 2.271±0.005 Å for the standard. No higher order shells are seen in cells while for the pyrite FeS_2 large peaks are clearly visible of distant shells. The results confirm the above qualitative assessment derived from the near edge spectra.

We obtained SEM images of *D. vulgaris* cells. From the images, it is clear that the cells grown in the presence of a higher concentration of iron in the solution have precipitates on the membrane. All the excess free iron in the solution was washed away thoroughly using 5 mM HEPES and

hence the mineral shown in the image is very likely the iron sulfide that precipitated on the cell membrane.

The results presented in this study show that dielectric spectroscopy can differentiate between abiotic and biotic mineralization under controlled laboratory conditions. An increase in the concentration of iron in the growth medium presumably increased the amount of iron sulfide precipitated on the cell membrane. Our SEM images suggest that higher iron concentrations in the growth medium led to greater iron sulfide precipitation on the cell membrane. We assume that the cells will take up as much metal as possible, eventually forming biominerals of iron sulfide that precipitates around the cell membrane.

We can use our theoretical model to describe the results. Our model assumes a cell to be a conducting sphere surrounded by a thin shell. As the cell membrane thickness increases, the dielectric permittivity decreases at low frequency. These cells have membrane potential of -80 to -140 mV across a 5 nm membrane. This causes polarization across the cell membrane in the β regime (see supporting information). Assuming membrane potential stays constant throughout the experiments, an increase in the membrane thickness would decrease the polarizability across the membrane, resulting in a decrease in dielectric permittivity. The value of relative dielectric permittivity of pyrite is around 10 and that for the cell membrane is between 2 and 10. These values are very close to each other and hence we could consider the membrane and the iron precipitate as a single shell, justifying use of our model. According to this model, cells grown in the presence of 0.0 g/l excess iron will have lowest membrane thickness and the cells grown in the presence of maximum concentration of excess iron will have highest membrane thickness. Thus the dielectric permittivity of the samples should decrease with an increase in the concentration of excess iron added to the growth medium. Previous TEM imaging, obtained by another group, of a cross sectional a single *D.*vulgaris cell with ZnS and FeS precipitates bound on its membrane clearly shows the mineral precipitate around the cell membrane. Our results are therefore consistent with the theoretical predictions of this model.

Previous studies have shown that for *Escherichia coli* cells, the dielectric permittivity is directly related to membrane potential at low frequency. Thus the decrease in the dielectric permittivity of cell suspensions with an increase in metal concentration might also be attributed to changes in membrane potential of the cells. However, we assume that the membrane potential will remain constant at very low mineral precipitation. Once the biomineral formation increases, it is difficult to predict the state of the cells. They could become weak or die. This would change the membrane potential of these cells and that in turn will change the dielectric permittivity of the samples. Thus we worked in the lower range of biomineralization. It has also been shown that membrane potential of cells changes with their growth stage. In our experiments, the growth stage was maintained the same for all cell measurements. As all the cells were grown for 48 hours, we assume that we were measuring healthy cells and that their membrane potential

remained unchanged. As the membrane potential for these cells is constant, we are confident that we are sensing the β regime which shows changes in the membrane thickness.

It can also be seen from our measurements that, for maximum concentration of iron (0.15 g/l Fe) in the solution, the curve for ε is very close to that of 0.05 g/l Fe. This could be explained by the assumption that cells reach a saturation limit as far as the amount of iron intake is concerned and the resulting biomineral concentration saturates. Anything measured beyond 0.15 g/l Fe is very likely biominerals instead of cells and the iron sulfide may then be abiotic. The SEM images show a large amount of biomineral formation for cells grown in presence of 0.05g/l iron as opposed to 0.0g/l iron.

The size of polarizable particles affects the frequency dependence of SIP measurements. The smaller the size of the particle, the higher is its surface area and the higher is the relaxation frequency. In our case, the difference in dielectric response is not just due to difference in the size of cells and pyrrhotite particles but due to their biotic versus abiotic state.

Here, we have shown that DS can be used to differentiate between biotic and abiotic iron sulfide minerals that evolve during bioremediation relevant perturbations. We have also shown that DS can detect changes in nano-scale thicknesses of biominerals formed around the cell membrane of sulfate reducing bacteria. These results provide information that could be used to improve interpretation of SIP data acquired in field studies of biodegradation experiments and to better understand biogeochemical processes associated with remediation treatments under laboratory conditions. We also show the potential of using DS as a novel laboratory technique for non-invasively the tracking progress of biomineralization experiments and knowing when to perform invasive sampling techniques.