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Intracellularly grown gold nanoislands as SERS substrates for monitoring chromate, sulfate and nitrate localization sites in remediating bacteria biofilms by Raman chemical imaging

Sandeep P. Ravindranath[†], Ulhas S. Kadam[†], Dorothea K. Thompson[‡], and Joseph Irudayaraj^{†,*}

Sandeep P. Ravindranath: psandeep@purdue.edu; Ulhas S. Kadam: ukadam@purdue.edu; Dorothea K. Thompson: dthomps4@utk.edu; Joseph Irudayaraj: josephi@purdue.edu

[†]Bindley Bioscience Centre, Purdue University, West Lafayette, IN 47906, United States of America

[†]Birck Nanotechnology Centre, Purdue University, West Lafayette, IN 47906, United States of America

[†]Department of Agricultural & Biological Engineering, Purdue University, West Lafayette, IN 47906, United States of America

[‡]Department of Biological Sciences, Purdue University, West Lafayette, IN 47906, United States of America

Abstract

Understanding the chemical composition of biofilm matrices is vital in different fields of biology such as surgery, dental medicine, synthetic grafts and bioremediation. The knowledge of biofilm development, composition, active reduction sites and remediation efficacy will help in the development of effective solutions and evaluation of remediating approaches prior to implementation. Surface-enhanced Raman spectroscopy (SERS) based imaging is an invaluable tool to obtain an understanding of the remediating efficacy of microorganisms and its role in the formation of organic and inorganic compounds in biofilms. We demonstrate for the first time, the presence of chromate, sulfate, nitrate and reduced trivalent chromium in soil biofilms. In addition, we demonstrate that SERS imaging was able to validate two observations made by previous studies on chromate/sulfate and chromate/nitrate interactions in *Shewanella oneidensis* MR-1 biofilms. Additionally, we show a detailed Raman mapping based evidence of the existence of chromate-sulfate competition for cellular entry. Subsequently, we use Raman mapping to study the effect of nitrate on chromate reduction. The findings presented in this paper are among the first to report- detection of multiple metallic ions in bacterial biofilms using intracellular SERS substrates. Such a detailed characterization of biofilms using gold nanoislands based SERS mapping substrate can be extended to study cellular localization of other metallic ions and chemical species of biological and toxicological significance and their effect on reduction reactions in bacterial biofilms.

*Corresponding Author: josephi@purdue.edu; Phone: 765-494-0388; Fax: 765-496-1115.

SUPPORTING INFORMATION AVAILABLE

SERS characterization of single cell based chemical maps in chromate reducing cells is presented. This material is available free of charge *via* the internet at ScienceDirect: <http://www.sciencedirect.com>

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Keywords

Surface-enhanced Raman spectroscopy (SERS); Chemical Imaging; Hexavalent Chromate; Sulfate; Nitrate; Bioremediation; *Shewanella oneidensis* MR-1

1. Introduction

Hexavalent chromium, is categorized as a priority pollutant and a carcinogen by U.S. Environmental Protection Agency (EPA). Due to its widespread use in manufacturing and processing industries it has been found at elevated levels in water and soil bodies near industrial area. Currently, very few chemical treatment approaches exist for chromate remediation, therefore adoption of bioremediative approaches offer more efficient means of achieving reliable removal of toxic Cr(VI) from groundwater as eco-friendly alternative. Several aerobic and anaerobic bacterial species have been found to be capable of reducing toxic and mobile Cr(VI) species to stable harmless Cr(III) complexes. *Shewanella oneidensis* MR-1, a facultative anaerobic Gram-negative bacterium has prompted interest in its applicability towards bioremediation due to capability in reducing chromate utilizing a vast repertoire of electron acceptors. Previously, studies have shown use of MR-1 as biological reducing agent for an array of compounds including Fe(III), Mn(IV), nitrate, nitrite, sulfate, chromate, U(VI), V(V), etc.

Biofilms, matrix-encapsulated bacterial population of cells adherent to each other are the most favorable way of bacterial life in environment. Extracellular polymeric substances (EPS), those enclose cells are a significant components of biofilms and play a major role as medium for interaction for bacteria with contaminating molecules [1, 2]. After reduction, contaminants tend to bind to cells or get trapped in bacterial EPS [3–6], which comprises of glycolipids, protein, nucleic acids and sorbed abiotic constituents [7].

Since majority of bacteria live as part of biofilms, it is vital to understand of bacterial life to recognize the effect of toxic chemicals on the fate of bacterial communities in the soil environment. Numerous bacterial species have been reported to form biofilms, and it seems clear that bacterial biofilm formation is favored under most nutrient-rich environments [8, 9]. The ability to form biofilms is considerably affected by the nutritional environment and chemotaxis [10–13]. Studies have indicated that microbial colonies may survive, communicate, and transfer charge through electrically conducting fibers known as ‘bacterial nanowires’ [14]. Studies in *Geobacterium* have shown a long-range charge transfer in an energy-efficient manner with a possible ten-fold increase in electricity production [15].

Biodegradation could be affected by numerous factors, such as soil pH, redox conditions, osmotic factors, toxic contaminants, and dependent on certain key co-substrates. As these factors are not uniformly distributed in nature, using the same bioremediative microbes and various approaches have got variable results [16]. Multiple studies have characterized chromate reduction in MR-1, but until now none have clearly identified an exact mechanism explaining this reduction, nor have addressed this question to such a high resolution [17–24]. An *in vitro* study, indicated the role of chromate reductase [25] in MR-1 and also proposed localization of these enzyme-reduction sites at the cytoplasmic membrane. In another study, Viamajala *et al.* investigated the effect of denitrification on Cr(VI) reduction in MR-1 [22] and demonstrated that specific rates of Cr(VI) reduction were inhibited by nitrates in the solution, and chromate inhibited nitrate reduction. Chromate (CrO_4^{2-}) anions, owing to its structural similarity to other inorganic anions such as SO_4^{2-} and PO_4^{3-} , has been known to enter cells via the sulfate transport system [26]. Chromate is also known to competitively

inhibit sulfate transport system as seen in *Pseudomonas fluorescens* [27] and in related gene expression studies [23, 28].

Thus, it is important to develop widely applicable analytical tools to study these complex interactions. Confocal Laser Scanning Microscopy (CLSM) coupled with Raman chemical imaging, is a robust method to characterize the biofilm structures. Unlike confocal microscopy, Raman imaging has potential to map high resolution spatial distribution of label-free biomolecules [29]. Additionally, Raman microscopy, as a molecular vibration-based spectroscopic technique offers the specificity needed for identifying the complex chemicals interactions with the EPS, within the biofilm. Besides specificity, peak shifts observed in Raman spectra are narrow enough to perform detailed multi-component analysis. Since Raman scattering is intrinsically weak, roughened surfaces of metals such as gold, silver or copper are used to enhance the signal. Surface enhanced Raman Spectroscopy (SERS) offers 10^7 – 10^{12} folds [30, 31] increase in signal enhancement and can be used to investigate vibrational properties of adsorbed molecules at very low concentrations [32, 33]. SERS has also been employed to enhance Raman signal of inorganic anions such as chromate [34], nitrate [35], sulfate [36], chlorate [37], and phosphates [38].

SERS imaging provides valuable spatial chemical information with image resolutions in the sub-micrometer range [24]. For successful bioremediation, knowledge of biofilm development, composition and active reduction sites and remediation efficacy within could help in the development of effective solutions and evaluation of remediative approaches prior to implementation. Here, we show a detailed Raman mapping based evidence for the existence of chromate-sulfate competition for cellular entry. Subsequently, we use Raman maps to study the effect of nitrate on chromate reduction. This detailed and characterized biofilm-gold nanoislands SERS mapping substrate can be extended to studying cellular localization of metallic and other chemical species and their effect on reduction reactions in bacterial biofilms. The same can also be applied to study drug or toxin interactions with biofilm in clinical devices.

2. Materials and Methods

2.1. Biofilm Cultivation

Biofilms were cultivated in mini-growth chambers (Nunc® clear flat bottom polystyrene 6-well plates). Gold coated glass slides (18 mm × 18 mm, EMF Corp, Ithaca, NY) were sterilized and placed into the growth chamber prior to inoculation. All biofilms were cultured in 0.25× LB medium. *S. oneidensis* MR-1 were grown in LB medium at 30°C to mid-log phase and incubated in 5 ml of LB broth at 0.01 OD and maintained under slow orbital shaking conditions (60 rpm). After an initial period of 12 h, 2.5 ml of growth media was replaced with fresh LB broth every 8 h for a period of 7 days prior to extracting slides for gold incubation [17]. Temperature of biofilms was maintained at a constant 30 °C.

2.2. Monitoring of Chromate, Nitrate and Sulfate and Gold Reduction in Reaction Medium

Biofilms (7 days old) cultured on gold slides were incubated in 5 ml of sterile PBS buffer (pH 7.4) containing reaction substrate 0.2 mM Cr(VI) / (0.2 mM Cr(VI) + 0.2 mM Sulfate) / (0.2 mM Cr(VI) + 0.05 mM Nitrate). Chromate, nitrate and sulfate concentrations were estimated using ChromaVer® 3, NitraVer® 5 and SulfaVer® 4 reagents from Hach Company, Loveland CO. Standard solutions were prepared using potassium chromate (K_2CrO_4), sodium nitrate ($NaNO_3$) and magnesium sulfate ($MgSO_4$) in LB broth (0.25 x). Chemical calibrations were performed on freshly prepared standard solutions and cellular reduction of analytes was estimated using UV-Visible measurements (Jasco Inc., Easton MD). Nanopure water (18.2 MΩ·cm) from a MilliQ water purification system was used for preparing all solutions. After 24 hour of incubation with reaction medium at 30 °C, the

slides were incubated in sterile PBS buffer containing 0.25 mM of HAuCl_4 (Au(III)). Biofilms were able to reduce gold chlorate ions into Au(0) within 24 hour as observed by the noticeable change in the color of the medium—colorless to bright pink. Slides were then washed with sterile nanopure water before drying at 37 °C [17, 21].

2.3. Confocal Raman Imaging and Spectroscopic Measurements

A SENTERRA confocal Raman system (Bruker Optics, Billerica, MA) fitted with a 785 nm laser, and a 50X air objective (N.A. 0.7) was employed for spectroscopic and mapping measurements. An integration time of 2 sec and a laser power of 10 mW (at source) were used to acquire Raman signal from biofilm samples. For mapping measurements, a $80 \times 80 \mu\text{m}^2$ grid (area) was chosen around the target biofilm cluster and the number of grids was selected to be 1600 resulting in a 40×40 grid on the cell and a step size of $2 \mu\text{m}$ with an individual grid size of $4 \mu\text{m}^2$. As the spatial resolution resulting with the 50X objective was $\sim 2 \mu\text{m}$, there were no overlaps between grids and the smooth transition between grids resulted in high resolution Raman intensity chemical maps of the formed biofilms [17].

2.4. Chromate Determination using ICP-MS

Inductively Coupled Plasma–Mass Spectrometer (ICP-MS) measurements were performed with a quadrupole ELAN DRC-e spectrometer (PerkinElmer SCIEX, Ontario, Canada), fitted with a Dynamic Reaction Cell (DRC) to determine the concentrations of trapped Cr(VI) and Cr(III). A PerkinElmer auto sampler model AS-93 Plus tray fitted with a peristaltic pump and a cross-flow nebulizer with a double pass spray chamber were used to deliver samples into the system. Identical instrumental conditions were maintained for the quantification of both total Chromium (Cr(VI) + Cr(III)) and reduced Cr(III). Biofilm samples set on the surface of gold squares were dissolved in 0.5% HCl by sonication and acid extract was used to estimate Cr(VI) and Cr(III) trapped within the biofilms. Chromabond $\text{NH}_2/3 \text{ mL}/500 \text{ mg}$ columns (i.e. Aminopropyl phase with a 3 mL volume and 500 mg of sorbent) were purchased from Macherey-Nagel (Duren, Germany). These columns were activated prior to separation of Cr(III) from Cr(VI).

2.5. Atomic Force Microscopic Measurements

AFM measurements were performed using a Dimension D3100 atomic force microscope (Veeco/Digital Instruments (DI), Santa Barbara, CA) equipped with a $\mu\text{Mash NSC36/AIBS}$ cantilever. Nominal stiffness of the cantilever was 0.95 N/m and the instrument was used in tapping mode. Resolution of the images acquired was set at 128 pixels. Images were processed using the WSxM v5.0 analysis software [39].

2.6. Confocal Fluorescence Lifetime Imaging Instrumentation

Fluorescence Lifetime measurements were carried out with a time-resolved scanning confocal microscope (Microtime 200, Picoquant GmbH, Berlin, Germany); with a 465 nm (40 MHz picosecond) pulse laser to excite the SYTO-9 labeled *S. oneidensis* MR-1 biofilms. The laser beam was focused onto the sample through an apochromatic 60X, 1.2 NA water immersion objective lens and the emitted fluorescence was acquired using the same objective and the excitation beam was subtracted using a dual band dichroic (z467/638rpc, Chroma). A $50 \mu\text{m}$ pinhole was used to reject the off-focus photons from the excitation volume, and the overall fluorescence was collected and separated accordingly using a dichroic beam splitter (600 dcxr, AHF, Chroma) and filtered by emission filters before being detected by the highly sensitive single photon detector (SPAD, single photon avalanche photodiodes) (SPCM-AQR, PerkinElmer Inc.) [40–42]. Biofilms were cultured on glass coverslips for these measurements under the same growth and gold reduction conditions as maintained for samples cultured on gold coated glass slides [17].

3. RESULTS AND DISCUSSION

Biofilm growth represents a protected form of microbial growth and biofilms are reported to have higher resistance to Cr(VI) toxicity than cells in suspension phase [5, 43]. In order to obtain detailed information on the biofilm matrix and to assess the influence of environmental factors on biofilm formation and development, particularly on the composition of EPS and reduction efficacy, sensitive analytical tool for rapid *in situ* biofilm characterization is necessary. This study has two key objectives: First, to characterize the intracellular gold nanoisland-based SERS imaging platform to understand chromate reduction and localization in MR-1 biofilms. Second, to investigate the effects of SO_4^{2-} and NO_3^- on chromate reduction and localization in MR-1 biofilms.

3.1. SERS Signal Characterization of Chromate, Sulfate and Nitrate

Raman spectra of metal ions such as chromium oxides [34, 44–46], lead oxides [47], mercury sulfides [47, 48], zinc oxides [47, 49] are available in literature and their spectra are well characterized. Recurrently, the majority of SERS analytes typically used are organic and very few studies have exclusively focused on SERS measurements of inorganic anions [50]. The low-frequency phonon-plasmon band at 220 cm^{-1} [33, 51] arising due to acoustic vibrations of surface features detected by the resonant excitation of localized plasmons are clearly evident in the spectra of Figure 1. The low-frequency Raman band is typically assigned to the Plasmon-phonon coupling originating from acoustic vibrations of surface characteristics selected by the resonant excitation of localized plasmons [51]. This band representing the metal characteristics was used to normalize the spectrum. SERS measurements for chemical controls including Cr(VI), Cr(III), SO_4^{2-} and NO_3^- as shown in Figure 1 are provided below. In addition to the low-frequency phonon-plasmon band at 220 cm^{-1} arising due to the acoustic vibrations [51] of surface features detected by the resonant excitation of localized plasmons, SERS signal of Cr(VI) (red spectrum in Figure 1) obtained from K_2CrO_4 (deposited on gold nanoparticles) show three key peaks at 757 cm^{-1} , 805 cm^{-1} and 840 cm^{-1} . The key peak observed around 842 cm^{-1} can be assigned to the stretching mode of 'Cr – O' [52, 53]. SERS spectrum of Cr(III) (black spectrum, Figure 1) show two peaks at 547 cm^{-1} and 598 cm^{-1} . The primary peak seen at 547 cm^{-1} can be assigned to $\text{Cr}^{3+}\text{-O}^{2-}$ stretching, but is generally found to be weaker than those seen in $\text{Cr}^{6+}\text{-O}^{2-}$ [34]. Nitrate (green spectrum) and sulfate (blue spectrum) have Raman bands at 1061 cm^{-1} and 982 cm^{-1} , respectively. These bands are assigned to the symmetric stretching vibration modes, ν_1 , of these anions [35, 36]. Since sulfate is present as an electrolyte, several studies have reported its SERS spectrum and these were used as standards [54–56].

3.2. Biofilm Development and SERS Substrate Characterization

To examine the interaction of *S. oneidensis* MR-1 within biofilms, it was crucial to establish that simple biofilms can be grown on glass coverslips and gold coated glass slides. AFM can deliver physical and surface morphological information in a nondestructive manner [57]. AFM imaging studies were performed using the tapping mode on samples grown as described in the Methods section. Figure 2(a–d) shows detailed characterization of MR-1 biofilm on gold coated glass slides. Using WSxM 5.0 software, we obtained a 3D projection of a small section of a biofilm cluster for a more detailed examination of surface features. EPS embedded around the cell surface, particularly at the cell–substrate periphery can be seen in Figure 2b and 2d (projection of Figure 2c) [58]. Even after rinsing cells multiple times with sterile buffer, the majority of the extracellular materials remained attached. These substances are known to be composed of a heterogeneous mixture of DNA, polysaccharides, lipo- and glyco-proteins, and other macromolecules secreted by bacteria [59]. As the EPS layer is thin and colorless, it is not feasible to observe these features with wide-field optical microscopy [59].

After biofilm characterization, the next step in substrate evaluation was to confirm the presence of gold nanoislands within cells, which act as SERS substrates to detect molecules in close proximity within the complex environment. Biofilms were cultivated on glass coverslips and the cells were treated with Au(III) indicated by its mild pink color in the reaction mixture which is an indicator for gold nanoislands formation. The cells were then washed with sterile PBS, incubated with SYTO-9, a membrane-permeant fluorescent stain that can bind to nucleic acids in cells to result in fluorescence in the green channel (Ex/Em: 488/500) and then dried before Fluorescence Lifetime Imaging (FLIM). It is known that Au nanoparticles emit very weak fluorescence and the associated fluorescence lifetimes are very low compared to lifetimes associated with standard fluorophores, for example ~0.4 ns for gold and ~ 2.1 ns for GFP [17]. As FLIM is independent of photobleaching and autofluorescence, it was possible to identify spots with lower average lifetimes, i.e., spots where gold was present in addition to SYTO-9, as a confirmation for *in situ* presence. A comparison of FLIM images with TEM images provide presence of gold nanoparticles localized within cells [17], also demonstrates the versatility of FLIM [40, 60]. FLIM images obtained for samples with gold reduction were compared to those without gold reduction. As shown in Figure 3, the control sample (Figure 3a) has an even distribution of green fluorescence throughout the cell cluster, whereas the samples (Figure 3b & 3c) with gold reduction show scattered blue spots (indicative of lower lifetime in the spots, see lifetime scale bar) confirming the presence of gold nanoparticles within cells. Thus, AFM and FLIM imaging served to cross validate the formation of biofilm clusters and subsequent confirmation of the presence of gold nanoislands within cells in the biofilm.

3.3. SERS Detection of Intracellular Cr(VI), Cr(III) and Sulfate using Gold Nanoislands

Distinct Raman bands for Cr(VI), Cr(III) and SO_4^{2-} were detected in cells loaded with gold nanoislands. Spectral differences for these ions as characterized in Figure 1 were used as the basis for identifying these chemical species within the intracellular milieu. Figure 4a and Figure 4b show clear traces of Cr(VI) and Cr(III) respectively within the SERS signal obtained from chromate reducing biofilm samples. Figure 4c shows distinct peak at 982 cm^{-1} in the bacterial SERS spectrum similar to that observed in the SERS spectrum acquired from the biofilm. All mapping data were processed using OPUS 6.5 software to remove weak fluorescence background with baseline correction using 'Rubber-band method' described in our previous work [32, 33, 51]. In addition, to account for variability of enhancement, the spectral intensities were normalized with phonon-plasmon peak around 220 cm^{-1} as a self-referencing standard [51]. Furthermore, background subtraction to remove mapping data interference from peaks of biomolecules was done and compared to controls to show that the interference due to competing molecules can be filtered out and the species of interest can be identified in the biofilms (Figure 5m–p and 6m–p).

Spatially resolved confocal Raman intensity images were acquired to map Cr(VI) bioreduction in chromate reducing *S. oneidensis* MR-1 biofilm samples cells. Raman chemical images were obtained from these samples by drawing a rectangular grid of $80\text{ }\mu\text{m} \times 80\text{ }\mu\text{m}$ with a $2\text{ }\mu\text{m}$ step size resulting in non-overlapping spots of 1600 spectral spots. Raman intensity maps for the defined area above derived with particular peak positions are shown in Figure 4d–4g. A Raman peak shift observed at the low frequency band in the range $230\text{--}260\text{ cm}^{-1}$ due to the phonon-plasmon coupling effect [51] was used as an indicator for the formation and presence of gold nanostructures by bioreduction as shown in Figure 4d and established in our previous study [51]. This mode has also been used as a self-referencing standard for SERS quantification [51, 61].

While Raman intensities integrated for Cr(VI) peak in the wavenumber region of $837\text{--}864\text{ cm}^{-1}$ (Figure 4e) exposed clusters of hexavalent chromium (toxic Cr(VI)), Cr(III) peaks mapped in the $531\text{--}567\text{ cm}^{-1}$ range (Figure 4f) showed a number of Cr(III) spots within the

cellular clusters. After initial characterization with cellular clusters in biofilm substrates, SERS signal from single cells were also acquired. Single cell Raman Intensity maps acquired from control (no chromate) and chromate reducing samples are processed and presented in Figure S1, where the sulfate inhibition of the chromate Cr(VI) conversion to Cr(III) can be seen in single cell maps (Figure S1k and S1l). Intracellular distribution of Cr(VI) in sulfate treated cells is significantly higher than that of Cr(III) indicating the inhibitory role of sulfate on chromate reduction.

3.4. Chromate/Sulfate Competition Studies

Three different concentrations of sulfate (0.1 mM, 0.2 mM and 0.4 mM) were added to chromate (0.2 mM) reducing early-stage MR-1 biofilms. Cr(VI) and sulfate concentrations were measured at the time of incubation as well as the end of reaction using chromaver and sulfaver based colorimetric detection assays. It is known that chromate can enter cells through sulfate transport systems [27, 62] and thus when cells are provided with equal concentrations of sulfate and chromate, they could compete for intracellular entry resulting in lower availability of chromate for reduction reactions. In order to study this competitive uptake and effect on chromate reaction, chromate reducing cells were treated with different concentrations of sulfate as explained above. The resulting Raman intensity maps for phonon-plasmon band, Cr(VI), Cr(III) and sulfate moieties are plotted across four different columns in Figure 5. Each row represents the 4 regions of spectral interest within the same mapped area. Figure 5a–5d represents chemical maps for chromate reducing samples with 0.1 mM of sulfate. Trace amounts of sulfate were observed in the sulfate spectral range and between the chromium species- Cr(VI) and Cr(III) maps, the majority of chromium was in the reduced Cr(III) form. Raman maps, as shown in Figure 5e–5h, for cells treated with equal sulfate and chromate concentrations reveal increased number of sulfate clusters in the sulfate map and the chromium species are almost split equally between the Cr(VI) and Cr(III) maps. Raman chemical maps (Figure 5i–5l) for samples treated with higher sulfate concentrations at 0.4 mM, there is considerable amount of cellularly trapped sulfate as shown in Figure 5j and the majority of Chromium is in the form of Cr(VI). In control samples, plain MR-1 biofilms with gold nanoislands grown in the absence of externally introduced sulfate and chromate, chemical intensity maps (Figure 5m–5p) at the same scale do not show any significant peaks after having accounted for the background. These concentration based competition studies indicate a direct role of sulfate in chromate reduction reactions. While mapping data is not enough to conclusively prove the uptake mechanisms, our data presents for the first time an imaging based direct competition assay of chromate and sulfate chemical species in bacterial biofilms. SERS peaks for these anions vary by less than 3–5% when enhancement is provided by intracellularly grown nanoislands owing to the presence of multitude of biomolecules at the nanoisland surfaces. In order to account for this variation, mapping data were processed to assess the intracellular distribution (intensity bandwidth on X-axes Figure 5 and 6). Upon optimal processing of SERS mapping spectra of control samples (Figure 5m–5p and Figure 6m–6p) with normalization and background correction, the species of interest could be identified in the selected spectral bandwidths.

3.5. Chromate/Nitrate Competition Studies

Inhibition of chromate reduction in MR-1 cells by nitrates and nitrites has been observed by Viamajala *et al.*, [22]. Unlike the effect of sulfate on chromate reduction with chromate which is known to be based on intracellular uptake competition, nitrate affects chromate reduction by an enzymatic inhibition mechanism. In order to test the inhibiting effect of nitrate on chromate in our mapping platform, chromate reducing biofilms were treated with three different concentrations (0.01 mM, 0.05 mM and 0.1 mM) of nitrate. Raman chemical maps acquired from these samples were processed and plotted into four spectral regions of

interest – phonon-plasmon band, nitrate peak, Cr(VI) and Cr(III) peaks as shown in Figure 6. At lower nitrate concentrations (10 μ M), maps (Figure 6a–6d) reveal minute quantities of the signal in the nitrate channel (Figure 6b) and a majority of chromium was in the reduced Cr(III) form.

At 50 μ M (five times the initial of concentration nitrate), there was a significant increase in the number of spots detected in the nitrate channel and there was a weak inhibitory effect on chromate reduction as evidenced in the distribution of clusters observed in Cr(VI) and Cr(III) channels. At higher nitrate concentrations of 100 μ M, a noticeable increase (as shown in Figure 6i–6l) in cellularly trapped nitrates was observed along with an increase in the inhibitory effect of nitrate on chromate reduction, since the majority of chromate was in the toxic-Cr(VI) form. Chemical intensity maps (Figure 6m–6p) of control samples composed of plain MR-1 biofilms loaded with gold nanoislands but grown in the absence of externally introduced nitrate and chromates, do not show any significant peaks (at same intensity scale) after having accounted for the background. In natural systems, Cr(VI) is generally present in conjunction with other chemical species that may act as competitive substrates in bacterial mediated reduction processes. Hence the design of effective *in situ* remediation strategies necessitates a detailed understanding of complex interactions between the several environmental factors and co-contaminants. Particularly nitrates, which are commonly present in most contaminant sites, may play a key role in the implementation of successful remediation systems. The experiments presented in this paper are one of the first studies to detect multiple metallic ions in bacterial biofilms using intracellular SERS substrates. In addition to detection of chromate, sulfate, nitrate and reduced trivalent chromium in the same sample, this method was able to validate two observations made by previous studies on chromate/sulfate and chromate/nitrate interactions in MR-1 biofilms. SERS could be a valuable tool for the evaluation of remediative approaches prior to installation at an actual site.

4. Conclusion

We demonstrated a novel bioanalytical approach based on SERS for simultaneous mapping of chromate, sulfate and nitrate in *Shewanella oneidensis* MR-1 biofilms. The different components of the biofilm were characterized by TEM, AFM, and FLIM. Raman chemical imaging enabled by *in situ* enhancements due to intracellularly grown gold nanoislands revealed the presence of Chromate, Sulfate, Nitrate, and trivalent Chromium in biofilms for the first time. In addition to a multicomponent imaging platform development our study also demonstrates the Chromate-Sulfate and Chromate-Nitrate interactions as well as Chromate-Sulfate competition in these biofilms. The findings of this study provides a new promising platform for further exploration of the redox state of multiple ionic species in the environment. The approach described is rapid, sensitive and selective and affordable by most laboratories. In addition, our study demonstrates that multiple ionic states in the samples can be detected with reproducible results. We expect the approaches developed to have a significant effect in the direct determination of multiple species as well as in enhancing our understanding of the interactions of multiple chemical species in a complex bioremediating environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SERS signal characterization of chemical controls: chromate (Cr(III) and Cr(VI)), nitrate, and sulfate.

Figure 2. Tapping mode AFM images for biofilms cultivated on gold coated glass slides. (a) Cells show a near continuous distribution at the surface of gold slide. (b) - (c) Higher magnification reveals extracellular structures. (d) 3D projection of a high resolution AFM image reveals the heterogeneity in the extracellular polymeric substances and their coverage at the surface of cell and cell-substrate interface.

Figure 3. Fluorescence Lifetime Microscopic Images. (a) Control – MR-1 biofilm without gold nanoislands - grown in the absence of Au(III). (b)-(c) Bacterial clusters grown in the presence of Au(III) show diffuse low-lifetime blue spots within cells.

Figure 4.

SERS spectra acquired from MR-1 biofilms loaded with intracellular gold nanoislands.

Intracellular SERS spectrum showing (a) traces of Cr(VI), traces of Cr(III) (b) and traces of sulfate (c). Raman Intensity maps averaged across a particular region of interest showing signal in four regions of spectral interest – phonon-plasmon band (d), hexavalent chromium (e), trivalent chromium (f) and sulfate (g).

Figure 5.

Chromate / Sulfate competition maps. Raman intensity maps acquired from three different samples averaged over four regions of spectral interest. (a)-(d) 0.2 mM chromate and 0.1 mM Sulfate. (e)-(h) 0.2 mM Chromate and 0.2 mM sulfate. (i)-(l) 0.2 mM Chromate and 0.4 mM Sulfate. (m)-(p) Control – gold reduction in the absence of Cr(VI) and sulfate.

Figure 6. Chromate/Nitrate competition maps. Raman intensity maps acquired from three different samples averaged over four regions of spectral interest. (a)-(d) 0.2 mM chromate and 0.01 mM nitrate. (e)-(h) 0.2 mM Chromate and 0.05 mM nitrate. (i)-(l) 0.2 mM Chromate and 0.1 mM nitrate. (m)-(p) Control – gold reduction in the absence of artificially introduced Cr(VI) and nitrate.