An experimental study of Au removal from solution by non-metabolizing bacterial cells and their exudates

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Abstract

In this study, we examine the initial interactions between aqueous Au(III)-hydroxide-chloride aqueous complexes and bacteria by measuring the effects of non-metabolizing cells on the speciation and distribution of Au. We conducted batch Au(III) removal experiments, measuring the kinetics and pH dependence of Au removal, and tracking valence state transformations and binding environments using XANES spectroscopy. These experiments were conducted using non-metabolizing cells of Bacillus subtilis or Pseudomonas putida suspended in a 5 ppm Au(III)-(hydroxide)-chloride starting solution of 0.1 M NaClO\textsubscript{4} to buffer ionic strength. Both bacterial species removed greater than 85\% of the Au from solution after 2 h of exposure time below approximately pH 5. Above pH 5, the extent of Au removed from solution decreased with increasing pH, with less than approximately 10\% removal of Au from solution above pH 7.5. Kinetics experiments indicated that the Au removal with both bacterial species was rapid at pH 3, and slowed with increasing pH. Reversibility experiments demonstrated that (1) once the Au was removed from solution, adjusting the pH alone did not remobilize the Au into solution and (2) the presence of cysteine in solution in the reversibility experiments caused Au to desorb, suggesting that the Au was not internalized within the bacterial cells. Our results suggest that Au removal occurs as a two-step pH-dependent adsorption reduction process. The speciation of the aqueous Au and the bacterial surface appears to control the rate of Au removal from solution. Under low pH conditions, the cell walls are only weakly negatively charged and aqueous Au complexes adsorb readily and rapidly. With increasing pH, the cell wall becomes more negatively charged, slowing adsorption significantly. The XANES data demonstrate that the reduction of Au(III) by bacterial exudates is slower and less extensive than the reduction observed in the bacteria-bearing systems, and we conclude that Au reduction occurs most rapidly and extensively upon interaction with cell wall functional groups.

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1. INTRODUCTION

Interactions between aqueous Au and bacterial cell walls can play an important role both in the epithermal deposition of Au (Jean and Bancroft, 1985; Mycroft et al., 1995; Ran et al., 2002; Widler and Seward, 2002; Mikhlin et al., 2006) and in the extraction of Au from leaching solutions during Au ore processing (Boyle, 1979; Torma and Oolman, 1992; Bosecker, 1997; Ubaldini et al., 2000; Reith et al., 2005; Reith and McPhail, 2006). Bacteria can cause the precipitation of Au(0) nanoparticles from Au(I) and Au(III) solutions on and around bacterial cell walls (e.g., Beveridge and Murray, 1976; Kashefi et al., 2001; Lengke and Southam, 2005; Reith et al., 2006), although the mechanisms of precipitation are not well-constrained. Several groups have measured Au(I) and Au(III) removal from solution by bacterial adsorption reactions, finding that Au removal from solution is most extensive under low pH conditions (e.g., Niu and Volesky, 2000; Ran et al., 2002; Nakajima, 2003; Tsuruta, 2004). They attribute this removal to adsorption, perhaps representing the first step in nanoparticle formation.

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There have been a number of experiments conducted to examine the ability of actively metabolizing, Gram-negative bacteria to adsorb and precipitate Au(III) from solution (Karthaikyan and Beveridge, 2002; Lengke and Southam, 2005, 2006; Lengke et al., 2006a,b, 2007; Reith et al., 2006). Gold(0) nanoparticle precipitation from Au(III) solutions onto bacterial cell walls likely involves a two-step process (Lengke et al., 2006a). The first step includes passive adsorption of aqueous Au(III) complexes to a sulfur, nitrogen, or oxygen moiety on the bacterial cell wall (Niu and Volesky, 2000; Lengke et al., 2006a), followed by the creation of a metastable Au(I)-sulfide intermediate species on the cell wall (Lengke et al., 2006a). Most previous studies of Au adsorption and nanoparticle formation involve high Au:bacteria concentration ratios and supersaturated solutions (e.g., Beveridge and Murray, 1976; Southam and Beveridge, 1994, 1996), and it is unclear whether these results apply to Au:cell ratios more typical of natural systems. Additionally, bacteria have been shown to metabolically reduce Au(III) complexes to Au(0) (Jian et al., 2009; Reith et al., 2009). However, in low nutrient environments, most bacteria present may be non-metabolizing (Ehrlich, 1996). Therefore, it is important to understand the role of non-metabolizing cells in the geomicrobiological cycling of gold.

X-ray Absorption Fine Structure (EXAFS) experiments were conducted by Song et al. (2012) which place rigorous constraints on the form, valence state, and chemical environment of bacteria-associated Au. Using both X-ray Absorption Near Edge Structure (XANES) and EXAFS measurements of Au bound to biomass, Song et al. (2012) found that greater than 90% of the adsorbed Au, which began as Au(III) in solution, was reduced to Au(I) by non-metabolizing Bacillus subtilis and Pseudomonas putida cells. Song et al. (2012) also observed no Au(0) or Au–Au nearest neighbors, indicating that Au(0) nanoparticles were not formed in their experiments. Furthermore, although Au was present in their experiments initially as aqueous Au(III)-chloride species, chloride was not a constituent of the Au–bacterial surface complexes that formed. Their EXAFS data demonstrate that Au on the B. subtilis cell wall is present as Au(I) bound to a mixture of amine and carboxyl and sulfhydryl functional groups, with increasing relative importance of the sulfhydryl groups with increasing pH and with decreasing Au loading on the cell walls.

The objective of this study was to deconvolute and understand the first steps in bacterial cell wall adsorption and reduction of Au(III) and to determine to what extent bacterial exudates may influence the reduction and adsorption reactions. We investigated the non-metabolic interactions between Au(III) and the Gram-positive bacterial species B. subtilis, and the Gram-negative bacterial species P. putida. Both species are common soil bacteria, and the thermodynamic stabilities of proton and metal complexes on their cell walls are well-characterized (Fein et al., 1997; Borrok and Fein, 2005; Fein et al., 2005; Ueshima et al., 2008).

We performed batch Au removal experiments, using washed cells in systems absent of electron donors, under similar experimental conditions studied by Song et al. (2012). We also used XANES to examine Au reactions with bacterial exudates. This compliment of experiments was conducted in order to provide constraints on the initial Au(III) binding and reduction mechanisms, thereby elucidating the effects of bacteria on the speciation and distribution of Au in natural and engineered systems.

2. MATERIALS AND METHODS

2.1. Bacterial growth

Bacillus subtilis and P. putida cells were cultured aerobically at 32 °C in 3 mL of trypticase soy broth with the addition of 0.5% yeast extract, and incubated for 24 h. The bacteria were then transferred to 2 L of the same growth medium and incubated for another 24 h at 32 °C. After incubation, the bacteria were harvested by centrifugation at 8100g, and then rinsed five times with a 0.1 M NaClO4 electrolyte solution. After each wash, the bacteria were suspended in clean electrolyte, a bacterial pellet was again formed by centrifugation for five minutes at 8100g, and the supernatant was decanted. After the final wash, the wet mass of the bacterial pellet was determined by suspending the bacteria in clean electrolyte in a pre-weighed test tube and centrifuging the suspension for two 30-min intervals at 8100g, decanting the bacteria-free supernatant after each interval. In this study, we report wet bacterial mass, and the wet mass determined using this approach is approximately 6 times the dry mass of the bacteria (Borrok et al., 2004).

2.2. Au removal experiments

All of the Au removal experiments conducted in this study have been summarized for clarity in Table 1.

2.2.1. Batch removal experiments

The extent of Au(III) removal from solution by bacteria was determined using batch experiments where the initial Au concentration was 5 ppm (2.50 × 10−5 M) and the bacterial concentration was varied (0.0, 0.7, 1.0, 2.0, 4.0, 5.0, 7.0, 10.0 g L−1 for B. subtilis and 0.7 and 1.0 g L−1 for P. putida). While the Au and bacteria concentrations in our study are higher than are likely to be found in natural systems, the Au:bacteria concentration ratio is realistic. Experiments were conducted in triplicate with fresh bacterial cultures for each to ensure reproducibility. The diagrams in the study depict the data from each replicate. When not indicated specifically, the analytical uncertainty is approximately the size of the graph symbols. The suspended bacteria were viable before and after the experiment, but non-metabolizing, since no external electron donors or sources of carbon were included in the experiments. The ionic strength in all experiments was kept constant using 0.1 M NaClO4. A parent solution of aqueous 5 ppm Au(III) in 0.1 M NaClO4 was prepared from a 1000 ppm HAuCl4 standard reference solution. We adjusted the 5 ppm Au-bearing parent electrolyte solution to pH 7, using a Thermo Orion model 420A bench-top pH meter, suspended a known mass of bacteria in this solution, and divided the suspension into 10 mL volumes in 15 mL
Table 1
Experimental conditions.

<table>
<thead>
<tr>
<th>Biomass type</th>
<th>Biomass concentration (g L(^{-1}))</th>
<th>Aqueous solution</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no bacteria)</td>
<td>0.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Removal</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.7, 1.0, 2.0, 4.0, 5.0, 7.0, 10.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Removal</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Reversibility (change pH)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4) and cysteine (Au:cysteine molar ratio of 1:14)</td>
<td>Reversibility (addition of competitive ligand)</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>0.7, 1.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Kinetics</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Exudates</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Reversibility (change pH)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5 ppm Au in 0.1 M NaClO(_4)</td>
<td>Kinetics</td>
</tr>
</tbody>
</table>

Polypropylene test tubes. The solution pH of each 10 mL suspension was then adjusted to a desired starting pH, ranging for these experiments from pH values of 3–10, using small aliquots of 0.1–1 M HCl or NaOH, and the systems were allowed to mix thoroughly by end-over-end rotation at 24 rpm for 2 h. The pH of each experimental solution was monitored every 30 min, and adjusted if required using small quantities of 0.1–1 M HCl or NaOH. The final pH was measured after 2 h of total reaction time, and the suspension was then centrifuged, and the supernatant was filtered through a 0.45 μm disposable nylon filter. The filtered supernatant was stored at 4 °C for no longer than 3 days prior to analysis for total dissolved Au. The concentration of dissolved Au was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) with matrix-matched standards. The concentration of Au that was removed in each system was calculated by subtracting the concentration of Au remaining in solution at the end of the experiment from the original Au concentration at the beginning of the experiment.

Some of the batch Au removal experiments were conducted as kinetic experiments in order to determine the Au removal rate for each condition studied. Three experiments were conducted for each bacterial species using portions of the 5 ppm Au parent solution adjusted to pH 3, 6 and 7, and using approximately 5 g L\(^{-1}\) of either *P. putida* or *B. subtilis*. Each experiment was mixed thoroughly and samples were taken periodically for up to 34 h, centrifuged, filtered and then analyzed for Au remaining in solution using ICP-OES.

### 2.2.2. Reversibility experiments

The reversibility of the Au removal reaction was determined using two different experimental approaches: (1) by first equilibrating biomass with a Au-bearing solution under low pH conditions, where high extents of Au removal were observed, and then promoting desorption by adjusting the pH of these suspensions to higher values; and (2) by exposing biomass from low pH Au accumulation experiments to solutions containing cysteine, a molecule that forms highly stable aqueous complexes with Au under the experimental conditions, and thereby promotes Au desorption. In the pH reversal experiments, approximately 5.0 g L\(^{-1}\) of *P. putida* or *B. subtilis* were suspended in the 5 ppm Au parent solution, which was adjusted to pH 3 before adding the bacteria. The experimental system was mixed thoroughly for 2 h to allow the biomass to accumulate Au from solution, after which the suspensions were divided into separate test tubes, and the pH of the individual suspensions of both bacterial species were adjusted over the range 2.0–10.0 and allowed to react for an additional 2 h. These experiments were conducted for both bacterial species separately. In the cysteine reversal experiments, 5.0 g L\(^{-1}\) of *B. subtilis* was suspended in the 5 ppm Au parent solution, adjusted to pH 3 and mixed thoroughly for 2 h. Cysteine was added to these suspensions to achieve a final molar ratio of Au:cysteine of 1:14 (2.50 × 10\(^{-5}\) M Au; 3.38 × 10\(^{-4}\) M cysteine), then the pH of the suspensions was adjusted to approximately 6.2 and 8.4. Samples were taken, following the same procedures as described above, after 30 min of biomass exposure to the cysteine-bearing solution. The bacteria in these suspensions were separated by centrifugation at 8100 g, and the concentration of dissolved Au in the supernatant was determined using the same ICP-OES approach that was described above.

#### 2.2.3. Bacterial exudate experiment

We also conducted tests to determine whether Au(III) reduction could be caused by dissolved bacterial exudates only, without bacterial cells present. To produce exudate-bearing solutions, we suspended 20 g L\(^{-1}\) *B. subtilis* cells in 0.1 M NaClO\(_4\) at pH 3.3 and 5.8 for 2 h. The bacteria were then separated from the solution by centrifugation, and Au(III) from a 1000 ppm HAuCl\(_4\) standard reference solution was added to the supernatant so that it contained 100 ppm Au(III). The high Au concentration was chosen to facilitate XANES measurements, and so that the final Au:bacteria ratio (and hence the Au:bacterial exudate ratio) was similar to that in the bulk Au removal experiments that involved bacterial cells. The oxidation state of the Au in solution was measured using XANES after 2, 4, 6, 8, and 10 h of reaction time for the pH 5.8 system (with samples referred to as Au Ex pH 5.8), and after every two hours of reaction time up to 12 h for the pH 3.3 system (with this
conducted using a total of 5 ppm Au in 0.1 M NaClO₄. No bacteria were present in the experiment. All experiments were conducted using a total of 5 ppm Au in 0.1 M NaClO₄.

The pH dependence of the Au removal is similar to that observed for the adsorption of organic acid anions onto bacteria (e.g., Daughney and Fein, 1998; Fein and Delea, 1998; Maurice et al., 2004). In these studies, adsorption is responsible for aqueous anion removal, with decreasing...
adsorption with increasing pH. The extent of adsorption, in these cases, is controlled by the speciation of the both the cell wall and of the aqueous molecules. Cell wall surface charge is controlled by cell wall acid functional group deprotonation reactions of the form:

\[ R - \text{AH}^+ \leftrightarrow R - \text{A}^- + \text{H}^+ \]  \hspace{1cm} (1)

where R represents the bacterial cell wall macromolecule to which the functional group (A) is attached (Fein et al., 1997, 2005). With increasing pH, both the cell wall and the organic acid molecules become increasingly deprotonated and develop a net negative charge, leading to an increase in electrostatic repulsion between the bacteria and the organic acid anion. The increasing repulsion with increasing pH is responsible for the subsequent decrease in adsorption. Alternatively, a decrease in Au removal from solution by bacteria with increasing pH could be due to a decrease in the rate of the reduction kinetics with increasing pH, as is the case for Cr(VI) removal and reduction by non-metabolizing B. subtilis cells (Fein et al., 2002).

3.2. Reversibility experiments

Our reversibility experiments test whether Au removal is due to adsorption or internalization, and they help to determine whether equilibrium is attained during the Au removal experiments. The pH reversibility experiments indicate that the Au(III) that was removed from solution at pH 2.9 does not return to solution simply by changing the solution pH for either bacterial species (Fig. 3). Metal cations and organic acid anions exhibit rapid and complete reversibility of bacterial adsorption reactions (e.g., Fein et al., 1999; Fowle and Fein, 2000). Therefore, the results of our pH reversibility experiments strongly suggest that the removal of Au(III) that is documented in Fig. 1 is a more complicated process than simple adsorption onto bacterial cell wall functional groups. Possible explanations for the non-reversibility behavior that we observe include internalization or precipitation of the Au by the bacteria. It is also possible that the Au removal reactions are not complete after the 2 h experiment duration, and that the equilibrium state for the system is characterized by significantly higher extents of Au removal from solution than we observed after only 2 h. The negatively charged Au(III)-hydroxide-chloride aqueous complexes that exist initially in solution would likely adsorb only weakly and would be expected to desorb readily if adsorption of those species was responsible for the Au removal behavior. The lack of reversibility, therefore, may indicate a change in the speciation of the Au(III) on the bacterial cell wall, a reduction of the Au(III) to cationic Au(I), which may bind more strongly, or precipitation of Au(0).

To determine if the Au was being internalized within the bacterial cells upon removal from solution, reversibility experiments were conducted using a strong Au binding ligand, cysteine. Cysteine is an α-amino acid (\(\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{SH}\)) which can form highly stable aqueous complexes with some metals (Baker and Czarnecki-Maulden, 1987). The functional groups on cysteine are carboxyl, sulfhydryl, and amine groups with pK\(_a\) values of 1.2, 8.7, and 12.5, respectively (Zimmermann and Burda, 2009). Gold(I) forms highly stable aqueous cysteine complexes (Zucconi et al., 1979) and our cysteine reversibility experiment tests whether dissolved cysteine can out-compete the cell wall functional groups, causing Au to desorb. If Au is internalized, cysteine would have little effect in remobilizing cell-associated Au, or the effect would be gradual. We observed that the addition of cysteine causes the extent of Au removal to decrease from the nearly complete removal at pH 3.1, observed in the system prior to the addition of cysteine, to 24% at pH 6.4 and 14% at pH 8.3 (Fig. 4). Slightly more remobilization of Au was observed at pH 8.3 than 6.4 likely due to increased deprotonation of the sulfhydryl site on the cysteine molecule, and enhanced Au-binding capability, under the higher pH condition. In addition, the extent of Au removal from solution at pH 6.4 and 8.3 in the cysteine-free batch removal experiments was 25% and 11%, respectively, so the attainment of similar extents of removed Au in the cysteine reversal experiments suggests rapid and complete reversal in the presence of cysteine.
The cysteine reversibility experiments demonstrate that the Au that was removed from solution by interaction with the bacteria is easily and rapidly accessible to solution, and therefore is likely not internalized, but is held on the bacteria as an adsorbed species.

3.3. Kinetics experiments

Our kinetics experiments demonstrated that the extent of Au removal from solution was dependent on the duration of the experiment, with increasing amounts of Au removed from solution over time for both bacterial species (Fig. 5 for B. subtilis and Fig. 6 for P. putida). The removal kinetics were fastest at pH 3.0, with slower removal observed with increasing pH. The pH-dependence of the removal kinetics that we observed is similar to what was observed for Cr(VI) and Np(V) removal by bacteria (Fein et al., 2002; Gorman-Lewis et al., 2005), and indicate that the 2 h results depicted in Figs. 1 and 2 represent only a snapshot of the removal process and not an equilibrium state. This result likely explains the lack of reversibility in the experiments that involved pH adjustments only. The equilibrium concentration of Au removed from solution under higher pH conditions is significantly higher than the extent of removal depicted in Figs. 1 and 2, so approaching that state from supersaturation during the pH reversal experiment did not cause significant Au desorption. Conversely, the presence of cysteine alters the equilibrium state, and by forming aqueous complexes with Au, cysteine promoted significant extents of Au desorption.

3.4. XANES for Au–bacterial exudates samples

The XANES measurements of bacterial exudate solutions that contain dissolved Au(III) initially, with no bacteria present, at pH 3.3 and 5.8 over the 12 h of exposure are depicted in Fig. 7. The XANES spectrum of 100 ppm Au(III)-chloride solution at pH 3.3 reacted with the exudates extracted from 20 g L⁻¹ wet mass B. subtilis solution for 12 h is similar to the Au(III)-chloride solution pH 3.0 standard (Fig. 8a). The XANES of 100 ppm Au(III)-chloride solution at pH 5.8 reacted with the exudate solution exhibited reduction from Au(III) to Au(I) over the 10 h of the experiment (Fig. 8b). Linear combination fits (Fig. 8c) show a progressive reduction of Au(III) to Au(I) in solution. Conversely, analyses indicate that the oxidation state of the Au does not change over the 12 h of the experiment for the pH 3.3 samples.

These results indicate that at low pH, B. subtilis exudates are not capable of reducing Au(III) to Au(I) in the 2 h timeframe of our batch experiments. Therefore, under low pH conditions, Au(III) reduction must occur on the cell wall following Au(III) adsorption, as more than 90% of the adsorbed Au is present as Au(I) (Song et al., 2012). At higher pH, B. subtilis exudates alone can reduce Au(III) to Au(I), but the rate of reduction is slower than is observed when bacteria are present. In the pH 5.8 exudate-only experiments, over 70% of the aqueous Au remained in solution as Au(III) after 2 h mixing. Although Au removal from
solution in these experiments involved both Au(III) reduction to Au(I) and chloride removal, the order of these mechanisms cannot be determined from our data. However, the data are consistent with Au(III) being reduced on the cell wall at all pH values studied. For example, XANES analysis of the Au exposed to the bacterial exudate solutions with no bacteria present indicates that at pH 3.0 greater than 95% of the Au in solution after more than 12 h of exposure time was still present as Au(III), but when bacteria were present, more than 90% of the cell-bound Au was present as Au(I). At pH 5.8, however, the bacterial exudates do exhibit the ability to reduce aqueous Au(III) to Au(I). After 2 h of exposure time, 28% ± 5% of the Au(III) was reduced to Au(I), and after 10 h 63% ± 5% of the aqueous Au(III) was reduced (Fig. 7). In contrast, after 2 h of exposure to B. subtilis cells at pH 5.8, more than 80% of the Au was removed from solution (Fig. 1), and more than 90% of this adsorbed Au was present on the cell walls as Au(I) (Song et al., 2012). Clearly, during the first 2 h of the experiments, the presence of the bacterial cell walls greatly enhances both the rate and extent of reduction of Au(III) to Au(I) relative to the rates observed in cell-free, exudate-bearing solutions. However, in the 6, 8 and 10 h experiments, more than 50% of the Au(III) in the exudate solutions were reduced to Au(I), so over longer time periods than 2 h, exudate reduction of Au(III) may play a significant role in the distribution and reduction of Au in bacteria-bearing systems.

The reason for the difference in reducing abilities of the two exudate-bearing solutions may relate to the speciation of Au(III) in solution. Fitting results by Song et al. (2012) indicate that below pH 5 the dominant Au species in solution is AuCl₄⁻, while at pH 7 the dominant Au species is Au(Cl₃OH)⁺. Our results suggest that the bacterial exudates that we have isolated can reduce Au(III) when it is present as a mixed Au-hydroxide-chloride aqueous complex much more efficiently than when it is present as AuCl₄⁻. The adsorption of either AuCl₄⁻ or Au(Cl₃OH)⁺ onto bacterial cell walls, however, represents a faster reduction pathway than occurs in exudate-bearing solutions, possibly due to the removal of chloride from the environment of the Au atoms that accompanies adsorption of the Au onto the bacterial cell walls.

3.5. Au-sulfhydryl modeling

Jian et al. (2009) and Reith et al. (2009) both observed that bacteria are able to metabolically reduce aqueous Au(III) complexes to Au(0) nanoparticles via passive accumulation followed by the formation of intermediate Au(I)-S complexes on bacterial cell walls and the subsequent reduction of these complexes to Au(0) nanoparticles. Our results are consistent with this overall mechanism, although because the cells in our experiments were not actively metabolizing, complete reduction to Au(0) nanoparticles was not observed. Our experiments provide detailed observations of the intermediate sulfhydryl binding stage of the reduction process. The EXAFS data from Song et al. (2012) indicate that under the conditions of our cysteine reversibility experiment, approximately 85% of the Au on the cell wall is bound to sulfhydryl sites, therefore we can use the measured extent of adsorbed Au in the experiment to estimate the concentration of sulfhydryl sites on the bacterial cell.
wall. Potentiometric titrations have quantified acidity constants and site concentrations for binding sites on *B. subtilis* (Fein et al., 1997, 2005), however it is unclear which of these, if any, correspond to sulphhydryl sites. Sulphhydryl binding appears to be dominant under conditions of low metal loading, but the concentration of these sites is low and other, more abundant types of sites dominate at higher metal concentrations (Guiné et al., 2006; Mishra et al., 2009, 2010).

The Au distribution in the cysteine reversibility experiments is controlled by the following reactions:

\[ \text{Au}^{3+} + 2\text{H}_2\text{O} + \text{cysteine}^\ominus \rightarrow \text{Au}^{+} + 2\text{H}^+ + \text{cysteine}^{-1} \]

and

\[ \text{Au}^{3+} + \text{cysteine}^{-1} \rightarrow \text{Au}^{+} + \text{cysteine}^{-1} \]

where \( \text{cysteine}^\ominus \) represents the sulfhydryl site on the bacterial cell wall, and \( \text{cysteine}^{-1} \) represents the cysteine molecule, deprotonated at the sulfur site. The equilibrium constant for Reaction (2) is unknown, but we assume that it is equal in magnitude to that for Reaction (3), measured to be 11.11 by Zucconi et al. (1979). Furthermore, we assume that the acidity constant for the cell wall sulfhydryl site is equal to that of the sulfur group on the cysteine molecule, using 8.7 for both \( pK_a \) values (Zimmermann and Burda, 2009).

These reactions, along with mass balance constraints on total cysteine and Au in the system, and the measured pH and adsorbed Au concentrations enable us to calculate the total sulfhydryl site concentration on the cell wall using FITEQOL 2.0 (Westall, 1982). EXAFS analysis of the biomass sample from the 7 g L\(^{-1}\) pH 6.5 experiment conducted by Song et al. (2012) indicates that 85% of the adsorbed Au is bound to cell wall sulfhydryl sites, so we assumed that the same percentage of the adsorbed Au in the cysteine reversibility experiment is also adsorbed to these sites. Using these assumptions, the concentration of the Au adsorbed to the cells in the cysteine reversibility experiment was used to determine the sulfhydryl site concentrations at pH 6.4 and 8.4, yielding an average calculated site concentration of 13.9 ± 4.8 (1σ) μmol g\(^{-1}\) of bacteria. The sulfhydryl site concentration is significantly smaller than the concentration of the other sites on the bacterial cell wall. Fein et al. (2005), for example, determined the concentrations of four discrete cell wall sites on *B. subtilis*, with concentrations between 44 ± 13 and 112 ± 36 μmol g\(^{-1}\) of bacteria. EXAFS results demonstrate that sulfhydryl sites can dominate the adsorbed Au budget despite the relatively low concentration of these sites on the cell wall of *B. subtilis*. Our results suggest that these low concentrations of sulfhydryl sites on bacterial cell walls can play an important role in controlling speciation and reduction of Au.

\[ 4. \text{CONCLUSION} \]

In this study, we use evidence from bulk removal measurements, reversibility studies, kinetics measurements and XANES to elucidate the initial steps involved in Au adsorption and reduction by bacteria and their exudates. Our experiments begin with aqueous Au(III), present in solution as negatively charged Au-chloride-hydroxide complexes. The strong pH-dependent behavior illustrated in Fig. 1 is likely controlled by electrostatic repulsion between the cell wall and the anionic Au(III) aqueous complexes. Although bacterial cell walls are negatively charged at low pH, the extent of electronegativity decreases significantly with decreasing pH. Our data indicate that below approximately pH 5–6, the electrostatic repulsion between cell wall and anionic Au(III) aqueous complexes is low enough to enable extensive binding of the Au(III) onto sulfhydryl sites and/or onto positively charged amine or/and carboxyl sites.

Our measurements of the valence state of Au in solutions that contain bacterial exudates only with no cells present indicate that while the exudates can cause some reduction of Au(III) to Au(I) under circumneutral pH conditions, the reduction is not extensive or rapid enough to explain Song et al.’s (2012) observation of the reduction of greater than 90% of the adsorbed Au(III) to Au(I) when bacteria were present. The rate and extent of Au adsorption is greatest under low pH conditions, and the pH dependence depicted in Fig. 1 likely reflects the pH dependence of the adsorption kinetics. Our pH reversibility experiments suggest that at equilibrium, nearly all of the Au is bound to the cells, regardless of solution pH. Both the Gram-positive *B. subtilis* cells and the Gram-negative *P. putida* cells adsorb nearly identical amounts of Au(III) and do so at nearly identical rates. Similarly, Song et al. (2012) found that both species exhibit similar capacities to reduce adsorbed Au(III) to Au(I). Therefore, it is likely that a wide range of bacterial species behave similarly, and that the adsorption and reduction steps that bacterial cell walls promote represent common initial steps in the formation of Au nanoparticles from solution. Bacterial exudates may also play a role in Au reduction under high pH conditions, but our experiments indicate that reduction of adsorbed Au(III) on bacterial cell walls is more rapid. Both cell wall and exudate reduction of Au(III) can significantly impact the speciation and fate of Au in bacteria-bearing geologic systems.

\[ 4. \text{ACKNOWLEDGMENTS} \]

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\[ 4. \text{REFERENCES} \]


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