Chemical Geology 373 (2014) 50–58

Contents lists available at ScienceDirect

Chemical Geology

journal homepage: www.elsevier.com/locate/chemgeo

Characterization of sulfhydryl sites within bacterial cell envelopes using selective site-blocking and potentiometric titrations

Qiang Yu [a,*], Jennifer Szymanowski [a], Satish C.B. Myneni [b], Jeremy B. Fein [a]

[a] Department of Civil & Environmental Engineering & Earth Sciences, University of Notre Dame, Notre Dame, IN 46556, USA
[b] Department of Geosciences, Princeton University, Princeton, NJ 08544, USA

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form 16 February 2014
Accepted 22 February 2014
Available online 5 March 2014

Editor: Carla M Koretsky

Keywords:
Potentiometric titration
qBBr
Sulfhydryl
Bacterial cell envelope
Adsorption

ABSTRACT

In this study, a novel approach was developed to estimate the concentration and acidity constants of sulfhydryl sites within bacterial cell envelopes, and we apply the approach to compare sulfhydryl site concentrations of *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus cereus*, *Shewanella oneidensis* and *Pseudomonas fluorescens*. The experiments involved the selective blocking of sulfhydryl sites using a thiol-specific molecule, coupled with total site concentration comparisons of blocked and un-blocked bacterial samples by potentiometric titration measurements to determine sulfhydryl concentrations. All five species studied contained measureable concentrations of sulfhydryl sites, ranging from 16.6 ± 3.3 μmol/g for *B. cereus* to 33.1 ± 7.6 μmol/g for *S. oneidensis*. No significant difference was found between sulfhydryl site concentrations on Gram-positive species relative to those on Gram-negative bacteria. However, the proportion of sulfhydryl sites relative to the total sites on each species was the highest for the thermophilic bacterium *B. licheniformis* with 14 ± 3%, and the four mesophilic species exhibited an average of 8 ± 2%. All species contained sulfhydryl sites with a pK_a of 9.2–9.4, but *B. subtilis* and *P. fluorescens* exhibited significant concentrations of sulfhydryl sites with much lower pK_a values as well. Our results suggest that sulfhydryl sites are present in relatively low concentrations over a wide range of bacterial diversity, but that their concentrations are high enough to control the binding of metals onto bacteria under low metal-loading conditions.

1. Introduction

Due to the abundance of organic acid functional groups within bacterial cell envelopes (Beveridge and Murray, 1980; Fein et al., 1997), metal cations can readily adsorb onto bacterial surfaces, thereby potentially affecting the speciation, distribution, bioavailability and mobility of the metal in the environment (Beveridge and Murray, 1976; Templeton et al., 2001; Martinez et al., 2002; Borrok et al., 2005a; Li and Wong, 2010; Sheng et al., 2011). Previous research indicates that carboxyl, phosphoryl and amine groups are important binding site types within bacterial cell envelopes (Fein et al., 1997; Cox et al., 1999; Ngwenya et al., 2003; Jiang et al., 2004; Guiné et al., 2006). In addition to these site types, the importance of sulfhydryl groups in metal–bacteria interactions was identified and highlighted in recent studies (Guiné et al., 2006; Mishra et al., 2010, 2011; Kenney et al., 2012; Pokrovsky et al., 2012; Song et al., 2012; Hu et al., 2013). Mishra et al. (2010), using EXAFS spectroscopy to study the speciation of Cd adsorbed onto *Shewanella oneidensis* under a wide range of metal loading conditions, identified sulfhydryl groups as the dominant binding sites for Cd for systems in which the initial Cd:biomass ratio was 300 μg Cd/g of bacteria in wet weight. Carboxyl and phosphoryl bindings become more important in the adsorbed Cd budget with increasing Cd loading on the bacteria, but sulfhydryl sites represent a detectable binding site in systems with an initial Cd:biomass ratio of less than 10 mg Cd/g of bacteria (Mishra et al., 2010). Au(I)–sulfhydryl (Kenney et al., 2012; Song et al., 2012) and Hg(II)–sulfhydryl (Mishra et al., 2011; Hu et al., 2013) bindings have also been observed as dominant mechanisms of Au and Hg adsorption onto bacteria when metal:biomass ratios are in the range of several hundred μg metal/g of bacteria or less. The concentrations of metals such as Cd, Hg, Cu, Ni, Zn and Pb in uncontaminated surface and ground waters, and even in some contaminated systems, are low, typically ranging from ng/L to μg/L (Klavins et al., 2000; Murano et al., 2007; Gupta et al., 2009; Lopez et al., 2010; Cui et al., 2011). Typical bacterial abundances in these systems can reach levels of approximately 10^9 to 10^{10} cell/L (Cole et al., 1993; Basu and Pick, 1997), or a bacterial concentration of approximately 0.1 to 1.0 g (wet mass)/L, using a conversion factor of 10^{10} cells/g (wet mass). Therefore, the metal loadings on bacterial cells in even contaminated geological systems are probably lower than concentrations that can saturate the sulfhydryl groups within bacterial cell envelopes, suggesting that metal–sulfhydryl binding may represent the dominant adsorption mechanism on bacteria. Metal–sulfhydryl binding, therefore, may control the transport, speciation, toxicity and bioavailability of

* Corresponding author.
E-mail address: qyu@nd.edu (Q. Yu).

http://dx.doi.org/10.1016/j.chemgeo.2014.02.027
0009-2541/

metals in natural environments. For example, Hu et al. (2013) recently demonstrated that the reduction of Hg(II) to Hg(0) by *Geobacter sulfurreducens* was markedly inhibited by Hg(II) adsorption onto bacterial sulfhydryl sites, and that only the Hg(II) adsorbed onto the other, non-sulfhydryl sites on the bacteria is bioavailable. Despite the importance of sulfhydryl sites in controlling metal adsorption by bacteria under low metal loading conditions, the concentrations and acidity constant values of sulfhydryl sites within bacterial cell envelopes have not been well constrained, making it difficult to understand and predict the extent of metal-bacterial sulfhydryl binding that occurs in natural and engineered geologic systems.

Currently, the best available method to estimate the density and acidity constants of binding sites on bacterial cell envelopes is based on potentiometric titration and surface complexation modeling (SCM) (Xue et al., 1988; Fein et al., 1997; Cox et al., 1999; Pagnanelli et al., 2000; Ngwenya et al., 2003; Yee et al., 2004). Although potentiometric titrations provide a reliable means to derive total site concentrations, and have been used effectively to constrain the protonation behavior of cell envelope functional groups (Daughney and Fein, 1998; Ngwenya et al., 2003; Takahashi et al., 2005), the interpretation of potentiometric titration data to infer concentrations and acidity constant values for specific proton-active binding sites is model-dependent (Fein et al., 2005). EXAFS spectroscopy can be used to identify metal–sulfhydryl site binding (Sarret et al., 1998; Guiné et al., 2006; Mishra et al., 2009, 2010; Pokrovsky et al., 2012), but it is not capable of yielding values for the sulfhydryl site concentrations within cell envelopes or the acidity constants of those sites (Mishra et al., 2005). Commonly used methods for the analysis of small biological thiol molecules involve the selective derivatization of the thiols with thiol-specific labeling reagents, followed by separation of thiol derivatives from interferences and UV/vis or fluorescence measurements (Dalle-Donne and Rossi, 2009; Hansen and Winther, 2009). However, the separation technologies that are required in this approach, such as high-performance liquid chromatography (HPLC) (Fahey and Newton, 1987), are not suitable for micron-sized bacterial cells.

In this study, we use a novel method that couples selective derivatization of sulfhydryl sites with potentiometric titrations and surface complexation modeling in order to analyze the sulfhydryl concentrations and acidity constant values within bacterial cell envelopes. Monobromo(trimethylammonio)bimane bromide (qBBr, Fig. 1), a cationic thiol-specific labeling molecule, was selected as the blocking molecule for bacterial sulfhydryl sites within cell envelopes due to its fast reaction rate specifically with thiol moieties within bacterial cell envelopes (Kosower et al., 1979; Kosower and Kosower, 1987). Previous studies demonstrate that qBBr can effectively block thiol sites on the surface of various biological cells such as human red blood cells (Kosower et al., 1979), guinea pig spermatozoa (Huang et al., 1984) and human leukocyte antigen (Whelan and Archer, 1993). Despite the differences in surface structures between various cells, the reaction mechanisms between qBBr and various biological sulfhydryl sites are the same (Fig. 1) (Kosower et al., 1979; Radkowsky and Kosower, 1986). During the interaction between qBBr and a sulfhydryl site, the bromine atom of qBBr is replaced by the sulfur atom from a sulfhydryl site. Therefore, exposed sulfhydryl sites should form strong covalent bonds with qBBr. Since the reaction product is not proton active, the sulfhydryl sites blocked by qBBr on cell envelopes should be inert during potentiometric acid–base titrations. Thus, the change in total site concentrations on cell envelopes determined by potentiometric titrations of biomass with and without qBBr treatment should be a direct measure of the sulfhydryl site concentration. Recently, qBBr titrations were used by Joe-Wong et al. (2012) in order to probe the sulfhydryl site concentration within cell envelopes of *Bacillus subtilis* using fluorescence spectroscopy. This method yields total sulfhydryl site concentrations, but does not provide constraints on the acidity constants of the probed sulfhydryl sites. In this study, we test a related approach, using potentiometric titration experiments involving biomass with and without qBBr treatment to probe sulfhydryl site concentrations and acidity constant values for five bacterial species. We use the results to determine if differences in sulfhydryl site concentrations and acidity constants exist between bacterial species with different cell envelope compositions (Gram-negative vs Gram-positive and thermophilic vs mesophilic).

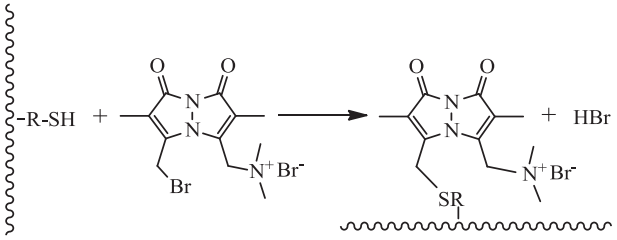

Fig. 1. Reaction of qBBr with sulfhydryl sites within bacterial cell envelopes.

2. Materials and methods

2.1. Bacterial cell preparation and thiol blocking reaction

In this study, all the bacterial concentrations are reported in terms of wet weight of the biomass. Five bacterial species were used in this study, including three Gram-positive bacteria (*Bacillus licheniformis, Bacillus subtilis* and *Bacillus cereus*) and two Gram-negative bacteria (*Shewanella oneidensis* and *Pseudomonas fluorescens*). Among these bacteria, *B. licheniformis* is a thermophile, while the other four species are mesophiles, although they were cultured under the same temperature in this study. The procedures for growth and washing of the bacterial cells were similar to those described previously (Fein et al., 1997, 2005). Briefly, all of the bacterial species were first cultured aerobically in 3 mL of trypticase soy broth with 0.5% yeast extract at 32 °C for 24 h and then transferred to 2 L of growth medium of the same composition at 32 °C for another 24 h. After incubation, bacterial cells in early stationary phase were harvested and rinsed three times with 0.1 M NaCl to avoid any unwanted interactions between qBBr and components of the growth medium. After rinsing, *B. subtilis* cells were stained using a LIVE/DEAD BacLight bacterial viability kit, which contains SYTO9 stain and propidium iodide, and then observed under a fluorescence microscope in order to test the integrity of the bacterial cells. While SYTO9 alone labels both dead and live cells to make them fluoresce green, propidium iodide penetrates only those cells with damaged membranes and reduces SYTO9 fluorescence, causing dead cells to fluoresce red. Our test results on *B. subtilis* cells showed a uniform green fluorescent color with no visible red cells, indicating that virtually all of the bacterial cells were still live and intact after the rinsing procedure.

Monobromo(trimethylammonio)bimane bromide (qBBr) and *N*-Acetyl-L-cysteine (A-CYS) were purchased from Santa Cruz Biotechnology, Inc. and Sigma-Aldrish, Inc., respectively. To block sulfhydryl sites, freshly prepared qBBr solution in 0.1 M NaCl was added to the bacterial suspension or to a 1 mM A-CYS solution, and the mixture was allowed to react for 2 h at room temperature under continuous shaking on a rotating plate at 60 rpm. The A-CYS molecules have two proton-active functional groups within the pH range that we studied: a carboxyl group with a pK_a of ~3.2 and a sulfhydryl group with a pK_a of ~9.5 (HSDB, 2014). Therefore, the titrations of the A-CYS solutions with and without qBBr-treatment serve as controls to test the ability of qBBr to block sulfhydryl sites and the selectivity of qBBr for sulfhydryl sites by determining if any of the carboxyl sites on A-CYS are blocked by the qBBr treatment. Previous studies indicate that the reaction kinetics between bromobimanes and sulfhydryl sites are pH-dependent and relatively rapid at pH 6–8 in the presence of a phosphate salt buffer (Kosower et al., 1979; Radkowsky and Kosower, 1986). For example, although human red blood cells are several microns in diameter,