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Quantifying adhesion of acidophilic bioleaching bacteria to silica and pyrite by atomic force microscopy with a bacterial probe

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ABSTRACT

The adhesion of acidophilic bacteria to mineral surfaces is an important phenomenon in bioleaching processes. In this study, functionalized colloidal probes covered by bioleaching bacterial cells (*Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*) were developed and used to sense specific adhesion forces to a silica surface and a pyrite surface in various solutions. Experimentally, recorded retraction curves of *A. thiooxidans* revealed sawtooth features that were in good agreement with the wormlike chain model, while that of *L. ferrooxidans* exhibited stair-step separation. The magnitudes of adhesion forces and snap-off distances were strongly influenced by the ionic strength and pH. Macroscopic surface properties including hydrophobicity and surface potential for bacterial cells and substrata were measured by a sessile drop method and microelectrophoresis. The ATR-FTIR spectra indicated the presence of different types of biopolymers on two strains of bacteria.

1. Introduction

The interactions between bacteria and minerals have been recognized for decades, which include bioleaching [1–3], bioflotation [4], formation of acid mine drainage [5] and biofilm [6]. Bioleaching is the process of recovering metals from sulphide ores using microbiological technology. The most important sulphur-oxidizer and iron-oxidizer involved in bioleaching industry are *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans* respectively [7]. The attachment of bacterial cells on mineral surface is essentially one of the most important aspects which enhance the metal sulphide dissolution in bioleaching processes, especially in the heap bioleaching [8]. Nowadays, bioleaching is widely practiced commercially and globally about 15% of the extracted copper comes from heap bioleaching [7]. However, due to a lack of comprehensive understanding of the microbiological processes, most hydrometallurgical plants are not operated under optimal conditions. Adhesion of bacteria to mineral surfaces can be affected by a variety of factors including pH, ionic strength and cell surface properties. To better understand the interfacial mechanism, knowledge on the adhesion forces between bacteria and mineral surfaces is required.

Various macroscopic approaches [3,9] and theoretical predictions [2,10] have been applied to measure and predict the adhesion of microorganisms to mineral surfaces. Atomic force microscopy (AFM) is a powerful tool for providing high-resolution images as well as probing interaction forces in biological area. AFM has been applied to visualize the biofilm on mineral surfaces [11–13], however only a few studies regarding quantitation of the interaction forces in bioleaching processes have been reported [1,14]. Additionally, the reported interactions were between the bacterial surface and AFM tip (e.g. silicon nitride) instead of the mineral surfaces [14].

Bacterial probes have been constructed by attaching a single cell to the AFM cantilever or forming a layer of biofilm [1,15–17]. However, some methods encounter problems such as low quality bacteria probes, in terms of incomplete coverage of the interaction position, and resulting in non-specific interactions between the cantilever surface and the substratum. Furthermore, most force measurements were carried out in solutions at neutral pH, where the positively charged polymers (e.g. poly-lysine) and adhesive proteins (e.g. polydopamine) can firmly attract live bacterial cells to the cantilever or substratum by electrostatic attraction [15,18,19]. However, in order to study the adhesion behaviour of acidophilic bioleaching bacteria, it is essential to carry out measurements in acidic solution (~pH 2), where the bacterial surfaces carry no net charge.

Based on the early work of Razatos et al. [20] and the colloid probe technique, we introduce a method for constructing

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bacterial probes which is capable of firmly attracting bacterial cells even in acidic solutions. The use of bacterial probes is a reliable and versatile platform for quantifying true adhesive interactions of bioleaching bacteria towards various mineral surfaces, particularly in solutions of low pH and high ionic strength. We also apply this technique to measure and compare the adhesion forces and snap-off distances of two key strains bioleaching bacteria.

2. Materials and methods

2.1. Bacteria strains and growth conditions

A. thiooxidans and *L. ferrooxidans* were kindly provided by Professor Guohua Gu (the Key Laboratory of Biometallurgy of Ministry of Education, Central South University, Changsha, China). *A. thiooxidans* was cultured in 9 K medium [21] with 3% (w/v) elemental sulphur. *L. ferrooxidans* was cultured in 9 K medium, and 4.47% (w/v) $FeSO_4$ was added for energy. All bacteria were incubated at 30 °C on a rotary shaker at 170 rpm.

2.2. Substratum preparation

The silicon wafers with a deposited thermal oxide layer of 100 nm thickness (Silicon Valley Microelectronics, USA) were cleaned by RCA SC-1 solution and stored in deionised water (Millipore, USA) before use [22]. The mineral samples were prepared from a piece of pyrite (~1 cm^3 embedded in an epoxy resin). The sample slice was manually polished with 3 μm and 1 μm diamond suspension. The sample disc was subsequently washed with copious amounts of deionised water before being cleaned with ethanol in an ultrasonic bath and with an UV/ozone cleaner prior to exposure to the bacterial probe. The root mean square roughness of freshly polished pyrite surface was measured using AFM (see Section 2.7) in contact mode at ten randomly chosen sites with triangular cantilevers (model NP, Veeco, USA) with a spring constant of 0.32 N/m.

2.3. Zeta potential measurements

The zeta potentials of bacteria were calculated from electrophoretic mobilities using Smoluchowski equation embedded in the software (ZetaPLUS analyzer, Brookhaven Instruments Corp., USA). Bacterial cells in the mid-exponential phase were harvested by filtering and then centrifugation at 10,000 × *g* for 15 min. The cell pellet was washed three times using sterile, deionised water to remove trapped ions. The washed cell pellet was re-suspended in deionised water, half-strength 9 K medium (1/2 9 K medium) of pH 6.15 and pH 2.05 respectively to obtain a final concentration of about 1×10^7 cells/mL. The suspensions were then transferred to the electrophoresis cell for measurement. For each sample, approximately 30 readings were taken from three independent experiments.

An EKA (electro kinetic analyser) with Clamping Cell (Anton Paar, GmbH, Austria) was used to obtain electrokinetic data for the planar silica surface. The ζ-potential was calculated according to the approach developed by Fairbrother and Mastin [23]. Streaming potential measurements were taken three times in each solution. The isoelectric point of pyrite surface was reported about pH 2, and the zeta potential of pyrite in deionised water is about −30 mV in literatures [24].

2.4. Contact angle measurements

Precipitate-free cell solution was filtered through Millipore filter paper (0.22 μm) using vacuum filtration to obtain a uniformly distributed cell layer on the whole area of the filter paper. The samples were air-dried and mounted on the glass slide with double-sided adhesive tape. The contact angles of bacterial lawn and mineral surfaces were measured by sessile drop method. A 0.5-μl droplet was illuminated by a fibre light from behind and observed using a ×10 objective (Nikon, Japan) and a progressive scan CCD camera (model XCD-SX910, Sony, Japan). An in-house Matlab code was applied to automatically calculate the dimensions of the droplet [25]. At least 5 drops of each solution were deposited onto substratum surface from three independent experiments and a total of 100 images were captured for each drop.

2.5. ATR FT-IR spectroscopy

The chemical composition of bacterial surfaces was examined by ATR FT-IR (attenuated total reflection Fourier transform infrared spectroscopy) using a Perkin Elmer Spectrum 100 spectrometer (Perkin Elmer Corp., USA) with an average of 43 scans in the range 650–4000 cm^{-1}.

2.6. Preparation of bacterial probes

The bacterial probes were fabricated by linking a microsphere coated by bacteria to the very end of a silicon nitride tipless cantilever. Nonporous silica microspheres (~20 μm in diameter, Fuso Chemical Corp., Japan) were cleaned by RCA SC-1 solution and then attached to the end of a tipless V-shaped cantilever (model NP-OW, Veeco, USA) with a very small amount of thermoplastic epoxy resin Epikote 1004 by means of a micromanipulator. The clean silica microsphere probes were functionalized with 1% (w/v) poly(ethyleneimine) (PEI, MW ~ 1300, Sigma–Aldrich, Australia) solution for 2.5 h, and the excess solution was decanted and the probes were rinsed in deionised water and stored at 4 °C [26].

Bacterial cultures in the mid-exponential phase were filtered and centrifuged at 10,000 × *g* for 15 min at 4 °C. The pellet was further washed in PBS buffer, and then resuspended in a 3% (v/v) glutaraldehyde solution for cell fixation at 4 °C for 2 h. After being fixed with glutaraldehyde, the cells were washed with PBS, and resuspended in PBS at 4 °C overnight. To immobilize the bacterial cells onto the functionalized silica particle, the colloid probe was lowered down with a micromanipulator to touch the cell suspension spread on a clean glass slide. Scanning electron microscopy (SEM) (Philips XL-30) was performed on all bacterial probes after AFM measurements to verify the presence of cells on the colloidal particle.

2.7. AFM force measurements

All force measurements were performed at a constant temperature of 22 ± 1 °C using a MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA) equipped with a closed fluid cell. The spring constant of each individual cantilever was calibrated by employing the thermal vibration method [27] embedded in the Asylum Research AFM software. The cantilevers used in the present set of data were found to have a spring constant of 0.07 ± 0.02 N/m. All force curves were recorded using a maximum loading force of 2 nN at a constant approaching/retraction velocity of 500 nm/s with a piezo movement of 6000 nm. At least two probes were used for each set of experiment at 2–3 contact locations per probe, as well as for the control (PEI-coated silica colloid probe). Under each experimental condition, more than 150 force curves were collected from at least two independent experiments. Force measurements were taken after each probe was immersed in a solution for at least 15 min.