Microporous and Mesoporous Materials 127 (2010) 32–40

Contents lists available at ScienceDirect

Microporous and Mesoporous Materials

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

Mechanistic study of the bactericidal action of silver-loaded chabasite on *Acidithiobacillus thiooxidans*

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ARTICLE INFO

Article history:
Received 19 February 2009
Received in revised form 17 June 2009
Accepted 21 June 2009
Available online 26 June 2009

Keywords:
Chabasite
Bacteria
Acidithiobacillus thiooxidans
Powder coating
Silver

ABSTRACT

The bactericidal action of silver-loaded chabasite against *Acidithiobacillus thiooxidans* was evaluated by measuring the variations in biogenic sulfate, ATP and biomass dry cell weight (DCW). The experiment was conducted using concrete specimens powder coated with 2.6% (Z-Ag-2.6%) or 18% (Z-Ag-18%) by wt. silver-loaded chabasite. Uncoated (UC) and blank chabasite-coated without silver-loading (ZC) served as control specimens. The growth of the bacterium was not hampered upon exposure to UC and ZC as confirmed by an increase in biomass DCW and cellular ATP. In Z-Ag-2.6%, cellular ATP declined to zero at the end of the experiment and the value of DCW did not increase from an initial value of 100 mg^{-1}. While there was an active biofilm in the UC with a maximum value of 0.1 mg DCW cm^{-2} and cellular ATP of 0.01 mg cm^{-2}, there was no significant biofilm development in all the zeolite coated specimens. In Z-Ag-2.6% and Z-Ag-18%, a progressive decrease in soluble silver (Ag-S) was observed due to uptake of silver by the bacteria as confirmed by the increase in particulate silver (Ag-P) from 0 to 4×10^{-2} mg Ag per mg DCW and 0 to 1.14×10^{-3} mg Ag per mg DCW, respectively. The half-life, $T_{1/2}$, for Z-Ag-18% was calculated to be 60 d compared to 700 d for Z-Ag-2.6%.

1. Introduction

Silver is one of the strongest antibacterial agents, with several oxidation states including zero valent (Ag^0), which is the most common, monovalent (Ag^+), and higher oxidation states (Ag^{2+} and Ag^{3+}), and has been known for its antibacterial activity since the times of ancient Greece [1]. In comparison with other antibacterial heavy metals, zero valent silver and silver ions are relatively less toxic to mammalian cells and tissues [2] and exhibit a broad spectrum of antibacterial activity at low concentrations [2–5]. Colloidal silver, a dispersion of tiny silver particles in water, is the form that is commonly used to generate antibacterial characteristics.

In recent years, there has been renewed interest by researchers, drug companies, food production, packaging and powder coating industries to investigate novel types of safe and cost-effective antibacterial materials; such as functionalized zeolites [6,7]. Zeolites are porous crystalline aluminosilicate minerals with uniform molecular-sized pores. They contain metal ions, such as calcium and sodium which are easily exchangeable by antibacterial metals such as silver, copper, and zinc ions for use as antibacterial agents [8–11]. The use of heavy metal-loaded zeolites as antibacterial agents in liquid medium has also been studied [12–15]. McDonnell et al. [16] demonstrated that zeolites exchanged with silver are highly hydrophilic and toxic to *Escherichia coli*, and reported that zeolite coating with a thickness of only 4–6 mm exhibited excellent adhesion to stainless steel and aluminum alloy for manned spacecraft application. Furthermore, the coatings were found to be extremely corrosion-resistant in strong acid and base solutions.

So far, neither has the longevity of silver and the extent to which it persists in its antibacterial activity been established nor is the mechanism of antibacterial action of silver ions, fully understood [4,5]. Two mechanisms are proposed for the bactericidal action of silver-loaded zeolites. One is the action of silver ion itself released from the zeolite matrix [17] and the other is that of reactive oxygen species generated from silver within the matrix [18–20]. While oxygen has been reported to be necessary for the bactericidal activity of silver-loaded zeolite by some researchers [21], functionalised zeolite has also been reported to be effective on oral bacteria under anaerobic conditions by other investigators [22]. It has been reported by researchers [17] that either the silver itself or the reactive oxygen species must interact with biological macromolecules, such as enzymes and DNA through an electron release mechanism for lasting antibacterial action. The main pathway of inactivation of macromolecules, such as enzymes is due to

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doi:10.1016/j.micromeso.2009.06.030

the formation of silver complexes with electron donors containing the main functional groups, i.e. thiols, carboxylates, amides, imidazols, indoles, and hydroxyls [18].

In our previous studies [7,23], we have demonstrated that liquid based epoxy-silver-loaded synthetic zeolite coating is antibacterial to *Acidithiobacillus thiooxidans* in suspension as well as in biofilm. Furthermore, we showed that the there was no detectable silver leaching from the zeolite matrix and leaching of metals such as zinc from the zeolite coating did not adversely impact wastewater sludge. In this paper, we present the application of *powder* coating to control biogenic concrete corrosion using *A. thiooxidans* as a corrosive agent. Silver-loaded natural chabasite was used as the antibacterial material. In addition to the growth kinetics of the aforementioned bacterium, the rate of release of silver from the chabasite matrix was investigated to estimate the service life of the silver-loaded chabasite coating. Mechanistic studies were undertaken to elucidate the mechanism of inhibition of silver.

2. Experimental

2.1. Specimen preparation

Unless specified, all chemicals were reagent grade and obtained from VWR Scientific (VWR Canada, Mississauga, ON). Ready mix concrete, known as Agilia Mix in the market was obtained from Lafarge Canada (Lafarge Canada Inc., London, ON, Canada). Cubic concrete specimens measuring $3.5 \times 3.5 \times 3.5\ cm^3$ were cut from $7.5 \times 7.5 \times 28\ cm^3$ concretes by a masonry diamond saw (Target Guardmatic TS 510). Mixing, casting, and curing of the concrete cubes were performed according to standard specifications (ASTM C192-90; C172-90, 2000).

2.2. Functionalization of natural chabasite

The chabasite used (GSA Resources Inc., Arizona, USA) had a mean particle diameter of 35 micron and a density of 1730 kg m^{-3}. Silver-loading of the chabasite was undertaken in the dark to avoid the photo-oxidation of silver by stirring a series of 20 g of chabasite samples in 500 ml of 0.05 or 0.01 N $AgNO_3$ solutions at 500 rpm in 500-ml closed polyethylene bottles placed in a constant-temperature water bath maintained at 60 °C for 4 days. The pH of each solution (5.0) was below the maximum natural precipitation limit, i.e. throughout the ion-exchange experiments, silver was in its monovalent form. Solid and liquid phases were separated by centrifuging at 5000 rpm for 5 min. Solid phase samples were washed with deionized water several times and dried in an oven at 105 °C. To ensure even distribution of data along the ion-exchange isotherm, the experiment was performed by varying the contact time of the chabasite particles and silver solution from 24 h to 96 h (data not shown).

2.3. Powder coating procedure

The powder coating mixture consisted of black epoxy powder, fluidizer (Al_2O_3), and natural chabasite (blank or silver-loaded). The fluidizer is the one used by the powder coating industry as an additive to uniformly distribute the antibacterial zeolite in the coating mixture [24]. Powder coating was performed at an epoxy to chabasite to fluidizer ratio (by wt.%) of 64:35:1. The epoxy used in the current experiment was a non-spherical black epoxy resin paint powder with an average diameter of 35 μm, provided by Links Syn Technologies Inc. (London, Ontario). The fluidizer powder was obtained from Evonik Industries (Essen, Germany). Before powder coating, the conductivity of the concrete specimens was improved by immersing them in a water bath at room temperature for 30 min. Uncoated concrete specimens (UC) and concrete specimens coated with unfunctionalized chabasite, without silver (ZC), were used as controls. The antibacterial efficacy of functionalized chabasite was assessed by coating the concrete specimens with either 2.6% (Z-Ag-2.6%) or 18% (Z-Ag-18%) by wt. silver-loading.

Fig. 1 shows the experimental powder spray system. Tests took place in a 750 mm (H) × 900 mm (D) × 750 mm (W) acrylic coating booth. A Nordson Surecoat corona spray gun (Nordson Corporation, TWGema, Amherst, OH, USA) was mounted on a support stand, directing the spray at the specimens. A mixture of the coating powders was transported to the gun manually and sprayed towards the specimens. A cone-shaped deflector was installed at the gun tip. The powder spray rate was controlled by controlling the air pressures of fluidizing air and atomizing air which was introduced into the spray gun fed with powder coating mixture. The corona charge generator was set at 50 kV, which is the usual operating voltage in industrial operation for powder coating. Coating was applied by spraying right on the moist concrete until the exposed surface area was covered with the coating mixtures by rotating the specimens support. The coated specimens were cured for 10 min at 200 °C in an oven (Sheldon Manufacturing Inc., 300N, Cornelius, OR).

2.4. Test microorganism and growth condition

A. thiooxidans strain ATCC 19703 was purchased from the American Type Culture Collection. The bacterium was grown in a basal nutrient medium (BNM) containing (in g l^{-1}): $(NH_4)_2SO_4$ (0.2), $MgSO_4{\cdot}7H_2O$ (0.5), $CaCl_2$ (0.25), KH_2PO_4 (3.0), $FeSO_4{\cdot}7H_2O$ (0.005) to which precipitated sulfur powder (10.0) was added. The ferrous sulfate heptahydrate was added as a solution, sterilized by filtration through a 0.22 micron-filter membrane to avoid oxidation to ferric sulfate during autoclaving. Sulfur powder was autoclaved for three consecutive days. The lumps of sulfur formed because of melting sulfur particles during autoclaving were aseptically crushed to powder using a sterile pestle and mortar in a properly sterilized laminar air flow hood (Air Clean 600 Work Station, Model 300, USA) before addition to the medium. The final sulfur powder added to the medium consisted of hydrophobic particles with an average diameter of 33.2 ± 5.4 μm. Separate batch experiments were undertaken to maintain the bacterium in its exponential growth phase for further studies employing the aforementioned medium.

2.5. Experimental procedures

2.5.1. A. thiooxidans growth inhibition experiments

The experimental setup utilized for growth assessment is illustrated in Fig. 2. All concrete specimens were separately glued on 5-cm diameter glass plate. The specimens were immersed in beakers filled with 350 mL of BNM inoculated with *A. thiooxidans*. In the inoculated experiment, 10% of the BNM was obtained from a previously sub-cultured two-week old *A. thiooxidans* medium with a dry cell weight of 50 mg l^{-1} ($\approx 10^4$ cells ml^{-1}). The beakers were closed by rubber stoppers with sampling ports, and sealed by 0.22 μm filter membrane to facilitate oxygen transfer from the air and preclude microbial contamination. The beakers were agitated using a Standard Shaker (Model Classic C10, New Brunswick Scientific, NJ, USA) at 150 rpm, to provide complete mixing as well as accelerate bacterial growth, and the working temperature was 20 ± 2 °C. The experiment was performed in a sacrificial mode. Control (UC and ZC) and test (Z-Ag-2.6% and Z-Ag-18%) specimens were placed in the beakers after the bacterium reached exponential growth phase (day-21).