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Antimicrobial Cu-bearing 2205 duplex stainless steel against MIC by nitrate reducing *Pseudomonas aeruginosa* biofilm

Jialin Liu[a], Ru Jia[a], Enze Zhou[b], Ying Zhao[c], Wenwen Dou[a], Dake Xu[b,*], Ke Yang[d], Tingyue Gu[a,**]

[a] Department of Chemical and Biomolecular Engineering, Institute for Corrosion and Multiphase Technology, Ohio University, Athens, OH, 45701, USA
[b] Corrosion and Protection Division, Shenyang National Laboratory for Material Sciences, Northeastern University, Shenyang, 110819, China
[c] Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518000, China
[d] Institute of Metal Research, Chinese Academy of Sciences, Shenyang, 110016, China

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ABSTRACT

In industrial and clinical settings, microbiologically influenced corrosion (MIC), also known as biocorrosion, is a major problem associated with materials degradation and infection. To mitigate biofilms, a Cu-bearing 2205 duplex stainless steel (2205-Cu DSS) was created by researchers to utilize the antimicrobial ability of copper. In this study, nitrate reducing *Pseudomonas aeruginosa* biofilm was grown as a nitrate reducing bacterium to investigate the antimicrobial efficacy and the MIC inhibition efficacy of 2205-Cu DSS under anaerobic condition. The results showed that both biofilm sessile cell count and electrochemically-measured corrosion rate were reduced compared with the 2205 duplex stainless steel (2205 DSS) control.

1. Introduction

Microbiologically influenced corrosion (MIC) is the corrosion induced by microbes (Muyzer and Stams, 2008; Jia et al., 2017b; Xu et al., 2017a; Zhou et al., 2018; Li et al., 2018). It occurs in the marine industry, the oil and gas industry, water utility systems, etc. (Li et al., 2016c; Lou et al., 2016; Jia et al., 2017a, 2017c; Liu et al., 2016, 2017). *Pseudomonas aeruginosa* was found to be corrosive against 2205 duplex stainless steel (2205 DSS), 2707 duplex stainless steel or even S32654 super austenitic stainless steel, all of which were widely used materials in some highly corrosive environments (Li et al., 2016b, 2017a; Li et al., 2017b; Xu et al., 2017b). In deep sea and oil pipeline environments, there is a lack of oxygen. *P. aeruginosa* can use nitrate or nitrite as terminal electron acceptor and reduce them to N_2 or NH_4^+ (Wu et al., 2005; Jia et al., 2017f). Elemental iron oxidation coupling nitrate reduction to N_2 or NH_4^+ is a thermodynamically favorable redox reaction (Jia et al., 2017g). This means *P. aeruginosa* has the thermodynamic driving force to be corrosive. However, the actual corrosion rate depends on corrosion kinetics. The corrosion rate will be negligible unless there is biocatalysis from an nitrate reducing bacterium (NRB) biofilm for nitrate reduction (Rodriguez et al., 2011) and for extracellular electron transfer (Xu et al., 2013).

P. aeruginosa is a Gram-negative facultative anaerobe which is widespread in seawater, and soils. Its relatively large genome encodes enzymes for antimicrobial efflux pumps and hydrolysis that make the organism resistant to antimicrobials such as penicillin, chlorine, glutaraldehyde, etc. (De Beer et al., 1994; Lister et al., 2009; Stover et al., 2000; Vikram et al., 2015). When a biofilm is formed, sessile cells in the biofilm are much harder to treat than planktonic cells (Jia et al., 2017h, 2017d). The extracellular polymeric substances (EPS) in the biofilm inhibit the diffusion of antimicrobials (Stewart, 1996). Sessile cells can slow down their metabolism to reduce the intake of antimicrobials (Tuomanen et al., 1986). It is also known that mixed-culture biofilms can form persister cells to survive antimicrobial attacks (Li et al., 2016d).

Copper has long been used as a disinfectant. Its antimicrobial efficacy depends on the released Cu^+ and Cu^{2+} (Grass et al., 2011; Sharifahmadian et al., 2013) near the copper surface. Cu-bearing 2205 duplex stainless steel (2205-Cu DSS) is a new antimicrobial metal (Nan et al., 2008). It has copper rich phases which are precipitated after the solution and aging treatments (Xi et al., 2016). Previous studies showed that, under aerobic condition, Cu-bearing stainless inhibited *P. aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (Lou et al., 2016; Nan et al., 2015; Sun et al., 2016) after they corroded the metal and released copper ions, which means a delayed antimicrobial effect. Anaerobic *P. aeruginosa* biofilm is more difficult to treat than the aerobic *P. aeruginosa* biofilm (Hill et al., 2005). In this study, the antimicrobial efficacy and MIC inhibition ability of 2205-Cu DSS against anaerobic *P.*

* Corresponding author.
** Corresponding author.
E-mail addresses: xudake@mail.neu.edu.cn (D. Xu), gu@ohio.edu (T. Gu).

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Table 1
Elemental compositions of 2205 DSS and 2205-Cu DSS (wt%).

Element	Si	Mn	C	P	S	Ni	Cr	Mo	Cu	N	Fe
2205 DSS	0.51	1.14	0.039	0.03	< 0.001	5.89	23.22	3.10	–	0.17	Balance
2205-Cu DSS	0.04	0.01	< 0.005	0.006	0.0034	6.03	23.63	2.90	3.02	0.23	Balance

aeruginosa biofilm grown as NRB was investigated for the first time for this new metal in comparison with 2205 DSS. Sessile cell count, biofilm thickness, biofilm images were analyzed. MIC corrosion was also investigated using several electrochemical methods.

2. Materials and methods

2.1. Coupons

2205 DSS was supplied by Taiyuan Iron & Steel (Group) Co. Ltd., Taiyuan, Shanxi, China. The 2205-Cu DSS used in this work was created by the Institute of Metal Research (IMR), Chinese Academy of Sciences, Shenyang, China as previously described (Xia et al., 2015). Table 1 lists the elemental compositions of the two metals. In this study, the metals were cut into coin-shaped coupons (10 mm diameter, 3 mm thickness). The coupons first underwent annealing treatment at 1050 °C for 1 h before they were quenched in room-temperature water. The coupons were then aged at 540 °C for 4 h and air cooled. The coupons were polished sequentially with 180, 400 and 600 grid abrasive papers. The coupons were painted with inert Teflon with only the upper surface exposed. Then, they were cleaned in an ultra-sonic water bath for 15 min followed by cleaning with pure isopropyl alcohol for another 15 min. The coupons were air dried under UV for 30 min in an anaerobic glovebox filled with nitrogen before use.

2.2. Bacterial strain and culture medium composition

The *P. aeruginosa* PAO1 strain (wild type) was donated by Prof. D. J. Hassett of College of Medicine at University of Cincinnati (Ohio, USA). Enriched artificial sea water (EASW) was used in this study as the culture medium. Its composition was (g/L): NaCl 23.476, $MgCl_2{\cdot}6H_2O$ 10.610, Na_2SO_4 3.917, $CaCl_2{\cdot}2H_2O$ 1.469, $NaHCO_3$ 0.192, KCl 0.664, KNO_3 0.625, KBr 0.096, $SrCl_2{\cdot}6H_2O$ 0.040, H_3BO_3 0.026, yeast extract 0.125 and tryptone 0.625. The pH of the culture medium was adjusted to 7.2 ± 0.1 before autoclaving for 20 min at 121 °C. Dissolved oxygen was stripped from the culture medium by sparging using filter-sterilized nitrogen for 45 min. Apart from this measure, 100 ppm (w/w) L-cysteine was added to the culture medium as an oxygen scavenger to cope with any possible oxygen ingress.

2.3. Antimicrobial efficacy assay

Anaerobic vials (Wheaton Industries Inc., Millville, NJ, USA) were filled with 100 ml medium and inoculated with 1 ml *P. aeruginosa* seed culture. The initial planktonic cell count in the 125 ml vials was 10^6 cells/ml. Three replicate coupons were placed in each vial before it was sealed and incubated without shaking at 37 °C for 7 days. After that, coupons were retrieved to count sessile cells in the biofilms on coupons using most probable number (MPN) method following a procedure described by Blodgett (2005). The NRB broth for the MPN enumeration of *P. aeruginosa* was supplied by Biotechnology Solutions (Houston, TX, USA).

2.4. Visualization of live/dead cells in biofilms

A confocal laser scanning microscope (CLSM) machine (Model LSM 510, Carl Zeiss, Jena, Germany) was employed to analyze the biofilms on coupons. A phosphate buffer solution (PBS) was used to rinse the biofilm on a coupon gently rinsed for three times. The Live/Dead® BacLight™ Bacterial Viability Kit L7012 (Life Technologies, Grand Island, NY, USA) was used to stain biofilms following a procedure reported elsewhere (Jia et al., 2017e). The excitation wavelengths of these two stains are 488 nm and 559 nm, respectively. Three spots on each coupon were picked to measure the biofilm thickness.

2.5. Visualization of biofilm morphology

A scanning electron microscope (SEM) machine (Model JSM-6390, JEOL, Tokyo, Japan) was used. The biofilm on a coupon was fixed with a biocide and then dehydrated before it was coated with a thin layer of palladium as described previously by Jia et al. (2017e).

2.6. Assay of released copper ions

One 2205-Cu DSS coupon and 3 ml freshly inoculated culture medium were added to a 10 ml anaerobic vial. It was incubated at 37 °C for 1, 7 and 14 days anaerobically. After incubation, each vial's contents were transferred to a 50 ml test tube and shaken for 1 min for mixing. The total concentration of copper ions Cu^+ and Cu^{2+} in the liquid was measured using an atomic absorption spectroscope (Model Z-2000, Hitachi, Tokyo, Japan).

2.7. Electrochemical measurements

Three hundred ml culture medium was added to a custom-made 450 ml three-electrode glass cell. A thin (10 mm × 10 mm) platinum sheet and a saturated calomel electrode (SCE) served as the counter electrode and the reference electrode, respectively. The test coupon was linked to a copper wire at the back that was covered with epoxy resin. The exposed coupon surface area was 1 cm^2. Electrochemical tests were performed using a potentiostat (Model VersaSTAT 3, Princeton Applied Research, Oak Ridge, TN, USA) with VersaStudio software version 2.44.4. Linear polarization resistance (LPR) was performed within the range of −5 mV to 5 mV vs. open circuit potential (OCP) at 0.167 mV s^{-1}. Electrochemical impedance spectroscopy (EIS) was recorded in the frequency range of 0.01 Hz–10^4 Hz. Potentiodynamic polarization curves were recorded within the range of −250 mV to 250 mV vs. OCP at 0.167 mV s^{-1}.

3. Results and discussion

3.1. Antimicrobial efficacy

Table 2 lists the sessile cell counts on the coupons after the 7-day incubation period. The sessile cell count on 2205 DSS was 4.6×10^8 cells/cm^2, much greater than 2.4×10^6 cells/cm^2 on 2205-Cu DSS. The results showed that 2205-Cu DSS achieved a 99.5% reduction in sessile cells compared to 2205 DSS.

Table 2
Sessile cell count data after the 7-day incubation in EASW inoculated with *P. aeruginosa*.

	2205 DSS	2205-Cu DSS
Sessile cell count (cells/cm^2)	4.6×10^8	2.4×10^6
Biofilm thickness (μm)	72.1 ± 5.6	50.9 ± 2.7