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USE OF SPUTTER-DEPOSITED 316L-STAINLESS STEEL ULTRATHIN FILMS FOR MICROBIAL INFLUENCED CORROSION STUDIES

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Abstract

Ultra thin films (12nm) were sputter deposited onto cylindrical germanium internal reflection elements pre-coated with a thin (2nm) layer of Cr_2O_3. Two crystals were inserted into Circle cell flow-through chambers and mounted on the optical bench of an Fourier Transform Infrared (FT-IR) spectrometer. One chamber was maintained as a sterile control while the other was sequentially inoculated with four bacterial species: *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Hafnia alvei*, and *Desulfovibrio gigas*, in that order. The water absorption band (1640cm^{-1}) was monitored and used to follow the deterioration of the ultra thin films. In this respect, the sterile control and inoculated films exhibited only slight differences during the 1000h course of the experiment. Assay of the visible biofilm that had accumulated on the surface of the inoculated crystal after 1000h revealed that the film incorporated viable cells from all four strains.

Introduction

In order to investigate microbial influenced corrosion (MIC) methodologies are required which can probe both fouling and corrosion processes as they occur, thus making the analysis of the interrelationships possible. It has been previously demonstrated that both bacterial colonization and deterioration of ultra thin films of copper deposited on germanium internal reflection elements (IRE) can be monitored in real-time using Fourier Transform Infrared (FT-IR) techniques [1]. In the present article some preliminary results are presented in which this methodology is adapted to the study of MIC of 316L stainless steel.

Sulfate reducing bacteria (SRB) are well-known to be instrumental in promoting pitting corrosion of mild steel [2], and are thought to promote corrosion of passive steels as well [3]. In order for SRBs to colonize a surface they need to be provided with an anaerobic reducing environment. It has been shown that *Desulfovibrio gigas* can colonize a stainless steel surface which has been exposed sequentially to three bacterial species: *Pseudomonas fluorescens*, *Bacillus subtillis*, and *Hafnia alvei* [4]. In research presented here we have initiated the establishment of this four member consortium (substituting *Pseudomonas aeruginosa* for *Pseudomonas fluorescense*) on ultra thin films of 316L which were deposited on germanium IREs. The impact of this four member consortium on the thin films was investigated using methodology we developed to study MIC of copper.

For our previous copper studies the films were vapor deposited onto the IREs. Vapor deposition is not suited for producing alloys of specified elemental composition because of the disparity in the melting temperatures of the component metals. A substantial part of the on-going effort in adapting our FT-IR technique to study of stainless steel involves learning to employ standard sputter deposition techniques in order to achieve ultra thin films of stainless steel which adhere to germanium substrates and which have an elemental

composition and crystal structure which matches the substrate. The films must also be of appropriate thickness in order allow the simultaneous monitoring of corrosion and fouling using the FT-IR technique.

Materials and Methods

Thin Film Deposition

Cylindrical Ge IREs were mechanically polished to 0.05μm and annealed at 873K for 2h in a vacuum furnace at a pressure of 5×10^{-9} Torr. Prior to deposition of the 316L film, the substrates were coated with a thin (2nm) layer of Cr_2O_3 deposited by RF-sputtering under an argon pressure of 30 mTorr. 316L films were deposited by DC-sputtering (argon pressure-30mTorr). Depositions were carried out in constant current mode and a fixed sample-to-target distance to attain deposition rates of 30 nm/ks. The base pressure was 2×10^{-5} Torr. Both targets were sputter-cleaned for over 15 minutes before deposition. IREs were slowly rotated in front of the target during sputtering in order to achieve a uniform coating. A calibration curve was constructed using profilometry measurements of deposited film thickness for various deposition conditions and thickness was monitored during deposition using a quartz crystal microbalance. New thickness measurements were performed on films deposited under the selected conditions. For this experiment stainless steel films were 12nm thick. Previous elemental analysis of 1μm films coated on glass substrates (obtained by energy dispersive x-ray spectroscopy in a scanning electron microscope) indicated that the elemental composition of thin films deposited by the protocol outlined above was almost identical to that of the target material [5]. In addition, crystal structure [5] and anodic polarization behavior in an abiotic environment [6], have been investigated.

FT-IR Measurements

Two stainless steel-coated cylindrical, germanium IREs were positioned in micro stainless steel, flow-through Circle cells (Spectra-Tech, Stamford, CT), and sterilized by ethylene oxide for 12h, then mounted on the optical bench of a Perkin Elmer Model 1800 double beam Fourier transform infrared spectrometer configured for continuous culture as described previously [1]. All FT-IR measurements were performed using a liquid nitrogen cooled, medium range mercury-cadmium-telluride detector. A background IR spectra was obtained in air for each element prior to introduction of sterile culture media. Spectra were collected at intervals from both circle cells as media flowed over the surface of the IRE and ratioed against the respective background spectrum (taken in air). A flow rate of 0.56mls/h was maintained except for a 24h period following each inoculation as described below. One circle cell served as a sterile control, while the other was inoculated with four different bacterial strains sequentially (described below). The stability of the stainless steel film in the presence of flowing culture media was evaluated by the area of the water absorption band at $1640 cm^{-1}$ (1740-$1540 cm^{-1}$). Experiments were performed in a temperature controlled atmosphere (23°C).

Microbiological

The culture media used was modified Hutner bacterial culture media composed of (per 1 liter dionized water, pH 7.4): yeast extract, 0.05g; sodium lactate, 0.05g; sodium succinate, 0.05g; ammonium nitrate, 0.05g; sodium sulfate, 0.12g; ferric chloride, 0.33ml of a 10mM aqueous solution; potassium phosphate, 5mM; 1ml of a 1 liter solution containing: nitrilotriacetic acid, 10.0g; $MgSO_4$, 14.45g; $CaCl_2$ $2H_2O$, 3.335g; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 9.25g; $FeSO_4$ $7H_2O$, 99mg; nicotinic acid, 50mg; thiamin HCl, 25mg; biotin, 0.5mg; 50ml

of metals "44" solution containing the following (per 100ml H_2O): EDTA, 250mg; $ZnSO_4$ $7H_2O$, 1095mg; $FeSO_4$ $7H_2O$, 500mg; $MnSO_4$ H_2O, 154mg; $CuSO_4$ $5H_2O$, 39.2mg; $Co(NO_3)_2$ $6H_2O$, 24.8mg; $Na_2B_4O_7$ 10 H_2O, 17.7mg; [7].

The four organisms used to establish a four member defined consortium biofilm on the stainless steel surface were: *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Hafnia alvei*, and *Desulfovibrio gigas*. Bacterial strain inoculums consisted of 1ml of a 48h culture. Flow through the Circle cell was stopped for 24h after each inoculation to permit colonization of the stainless steel surface. Times of each inoculum were as follows: *Pseudomonas aeruginosa*: 56h, 7 x 10^7 cfu/ml; 410h, 2 x 10^4 cfu/ml; 457h, 7 x 10^7 cfu/ml; *Bacillus subtillis*: 531h, 6 x 10^6 cfu/ml; *Hafnia alvei*: 577h, 6 x 10^7 cfu/ml; and *Desulfovibrio gigas*: 650h, 10^2 cells/ml, 823h, 10^6 cells/ml, 991h, 10^1 cells/ml. The viable cell counts in each inoculum have been given after each inoculation time. For aerobic bacteria viable cells in each inoculum were enumerated by plating on modified Hutner medium containing 1.5% Bacto agar. Viable cells of *Desulfovibrio gigas* in each inoculum were estimated by most probable number in modified Postgate's medium [8].

At the end of the experiment, each IRE was aseptically recovered from the Circle cell and swabbed with a sterile Q-tip and the bacteria transferred to sterile media, serially diluted, and the respective species enumerated as follows: total aerobic bacterial colony forming units were obtained by plating on modified Hutner medium containing 1.5% Bacto agar. *Pseudomonas aeruginosa* was enumerated as translucent colonies on Pseudomonas Isolation Agar. *Hafnia alvei* was enumerated as brick-red colonies on Mac Conkey's Agar. *Bacillus subtillis* was enumerated on Phenylethanol Agar. Presence of *Desulfovibrio gigas* was determined by the Conoco Rapid Chek™ II Assay based on adenosine phosphosulfate (APS) reductase activity. Viable cell counts can be estimated on the basis of the color intensity.

Results and Discussion

The film integrity was monitored by measuring the area of the water absorption band at 1640cm^{-1} (1540cm^{-1} to 1740cm^{-1}) during the course of the experiment (Fig.1). It has been previously shown that this provides a good index of film thickness for copper films deposited on germanium IREs [9]. The metal film decreases the penetration of the evanescent field into the adjacent aqueous media, resulting in a relative decrease in the water adsorption band compared to an uncoated IRE. Conversely, the water absorption band increases for films of decreasing thickness. Qualitatively, the same relation is expected for Ge crystals coated with other metals (i.e., stainless steel). Referring to Fig.1, immediately after exposure to flowing culture media (time=0h) the area of the water absorption band (taken from 1540cm^{-1} to 1740cm^{-1}) was 0.52% (.18) and 0.76% (.26) of its average value (34.5 ± 4.4) for a clean (uncoated) IRE for the inoculated and control circle cells, respectively. During the 1000h of the experiment, area of the water absorption band increased to 1.33% and 2.23% (of its average value for an uncoated IRE) for the inoculated and control cells, respectively, indicating a slight thinning of the films (Fig.1). These changes in the water adsorption band can be converted into approximate estimates of thinning of the film by referring to the theoretical reflectivity vs. film thickness curve for thin films of iron [10]. According to this estimate the decrease in average thickness of the deposited films during the course of the experiment was, respectively, .32nm and .58nm for the inoculated and control IREs. This indicates that stainless steel films on both the inoculated and control cells remained relatively intact.

At the end of the experiment both crystals were removed, visually inspected, and assayed for viable cells present on the surface of the crystals. Visual inspection indicated the

presence of a thick, diffuse, biofilm on the inoculated crystal. Appearance of black granular entrapped particles indicated the formation of iron sulfide had occurred. In contrast the control cell appeared to be almost entirely free of any deposits except for a small darkish area near the input port.

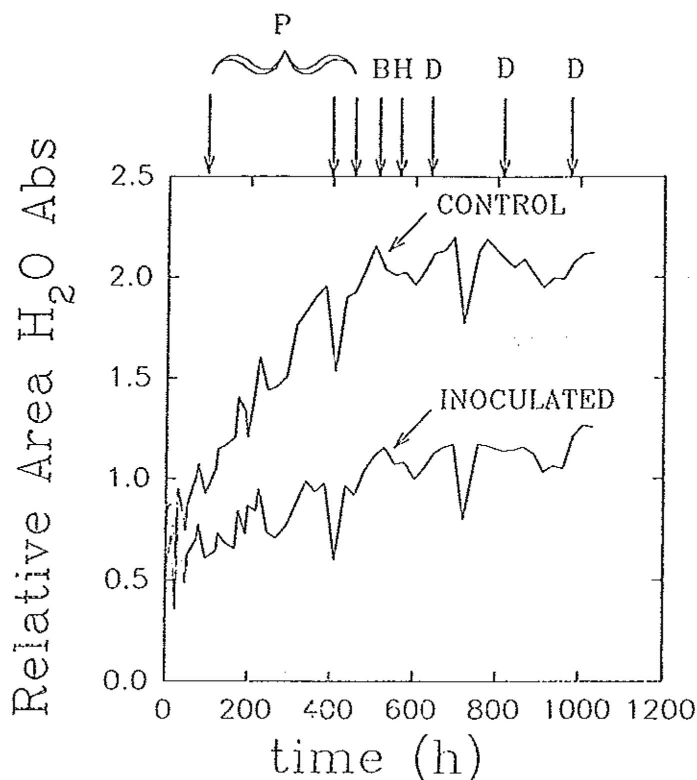

Fig. 1: Ordinate: area of water absorption band (1740–1540 cm^{-1}) normalized to area for a clean, uncoated crystal (%); abscissa: time in hours. times of inoculation are indicated above frame.

For the inoculated cell, counts were made for each of the four strains by using various selective and/or isolation media as described in the Methods section. The plate counts on selective media, together with the assay for APS reductase activity, indicated that all four strains had been recovered from the biofilm which grew on the crystal of the inoculated cell. The viable cell counts of each strain enumerated as described in the Methods section, as well as the total aerobic cfu's, are indicated in Table I.

Table I

Viable Cells Recovered from Inoculated Circle Cell Biofilm

Total[1]	*P. aeruginosa*	*H. alvei*	*B. subtillis*	*D. gigas*[2]
9.5±2.0 (10^5)	3.8±1.0 (10^5)	2.3±.05 (10^5)	1.0±.2 (10^5)	1.0 (10^5)

[1]total aerobic cfu
[2]enumerated by Conoco Rapid Chek™

The results presented here demonstrate that exposure of the surface of an ultra thin film of 316L stainless steel to the four bacterial species, *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Hafnia alvei*, and *Desulfovibrio gigas*, induces establishment of a four member

consortium biofilm. The presence of a healthy biofilm on the surface of the inoculated crystal was obvious both from visual inspection and the microbiological assay. However, any IR bands associated with this biofilm were undetectable, apparently buried in noise. The low S/N ratio resulted from two factors: the biofilm was patchy, covering only a part of the available IR adsorbing surface of the IRE, and the stainless steel film was relatively thick, allowing only a slight penetration of the evanescent field. Films that are only 2-3nm should still be continuous, and will allow substantially more penetration of the evanescent field. We have observed only slight deterioration of stainless steel thin films after a period of 1000h both for sterile and fouled films. This is encouraging in the sense that it indicates that our films are adherent to the germanium surface, and stable when exposed to flowing media and mixed species microbial biofilm. Future work will involve use of thinner films and longer experimental duration times. We expect to eventually observe an increased rate of thinning of films exposed to microbes, indicating corrosion, and IR bands associated with bacterial colonization of the surface.

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