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Short Communication

The fate of the protective oxide film on stainless steel upon early stage growth of a biofilm

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ABSTRACT

For metals and alloys that are protected against corrosion by a surface oxide film (the passive film), the modification or degradation of this passive film in presence of bacteria is a key issue in terms of integrity and reliability of structures. The objective of this work was to reveal the nature of the interaction of stainless steel with bacteria, in particular the modifications of the passive film after bacteria adhesion in the early stage of biofilm formation. An innovative approach, based on advanced surface analysis, has been developed. The use of Time of Flight Secondary Ions Mass Spectrometry (ToF-SIMS) allowed us to have access to and characterize the bacteria/substrate interface.

Here we show that a passive film remains under the bacteria, but its chemical composition is modified. The passive film under *Escherichia coli* bacteria, for a surface coverage by bacteria of ~20%, is enriched in Cr compared to the passive film on stainless steel not exposed to the bacteria.

1. Introduction

The interaction of materials with biological environments is a key issue for the lifetime of structural and functional materials [1–5]. Biofilms are complex structures consisting of an accumulation of micro-organisms contained within extra-cellular products, inorganic or organic debris attached to a surface [6,7]. Biofilm formation has been recognized as a widespread problem in design and operation of industrial equipments, such as heat exchangers, cooling water and food processing systems. Local modifications of the environment in terms of pH, redox potential, dissolved oxygen gradient or secretion of organic/inorganic species [8], can contribute to corrosion of metallic surfaces [9–11] and/or deterioration of products quality such as taste, odor and color. By protecting and/or supporting pathogenic microorganisms, biofilms also represent a major complication in implanted and percutaneous medical devices [12–18].

Due to their corrosion resistance properties, stainless steels are often used in such industrial fields. The composition, chemical states and thickness of passive films formed on stainless steels have been studied in details by X-ray Photoelectron Spectroscopy (XPS) [19–22]. SIMS has been used in a few studies of passive films, mainly on Fe–Cr binary alloys [23–25]. It is recognized that the corrosion resistance of stainless alloys is due to the formation of a thin passive chromium and iron oxide/hydroxide [17,26–29]. When bacteria adhere to stainless steel surfaces and form a biofilm, the exact fate of the passive film under the bacteria is still unknown [4,5], despite numerous studies that have shown the potential ennoblement of stainless steel exposed to bacteria [1,3,4,30–32]. There is so far no answer to the question: "is it the passive film or the metallic surface that is the preferred material for bacterial attachment?"

To address these key issues, a novel analytical strategy has been developed, based on a modern surface analytical technique: Time of Flight Secondary Ions Mass Spectrometry (ToF-SIMS). To have access to and characterize the bacteria/substrate interface in the early stages of biofilm formation (1 h exposure to bacteria), we used a dual beam ToF-SIMS with Bi^+ ions for analysis and Cs^+ ions for sputtering. Longer exposure times to the bacteria (24 h), more representative of a mature biofilm, have been done and will be the purpose of a forthcoming paper.

The bacteria used in this study are *Escherichia coli*. Although they are generally not considered to lead to corrosion (in contrast with e.g. sulphate-reducing bacteria on carbon steels), the issue of stability of the passive film in presence of *E. coli* is important, in particular for the use of stainless steels in the food industry, and this is the subject of this study.

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2. Experimental methods and procedures

2.1. Bacteria

The bacterial strain used in this study, *E. coli* K12, was kindly provided by the Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés from INSA Toulouse (M. Mercier-Bonin). The bacteria were stored at −196 °C in glycerol. A work stock was kept at −20 °C. Cells were subcultured in the same medium as the work culture Luria Bertani Broth (Biomérieux, France). Microorganisms were grown overnight at 37 °C under continuous agitation until the early-stationary phase culture. Then, bacteria were harvested by centrifugation (7000*g*, 10 min.), washed twice and resuspended in saline solution (NaCl 0.15 M) to have a final concentration of 10^8 cfu/mL (measured by plating).

2.2. Samples

AISI 316 L bright annealed stainless steel (Fe base alloy with 17% Cr, 10% Ni and 2% Mo in wt.%) from APERAM was used. The sample dimensions were $10 \times 10\,mm^2$. The detailed chemical analysis of the stainless steel is given in Table 1.

Prior to any testing, the samples were mechanically polished in order to obtain a mirror polish finish. Surfaces were then first degreased in an ethanol/acetone (50/50) (Prolabo, Rectapur®) bath for 5 min. The samples were then rinsed 3 times in distilled water at 50 °C for 1 min, and 5 times in distilled water at room temperature for 1 min. Coupons were dried and stored in sterile Petri dishes before use.

2.3. Bacteria adhesion experiments

AISI 316 L stainless steel coupons were immersed in the bacterial suspension in static conditions at 20 °C for 1 h. The suspending liquid was then eliminated. Non-sticking cells were removed by five successive rinses with the sterile saline solution (NaCl 0,15 M). Some coupons were only immersed in sterile saline solution without bacteria in the same conditions (1 h at 20 °C) and served as reference samples.

For epifluorescence analysis, adherent cells were labeled with a fluorochrome solution (DAPI, Molecular Probes, Invitrogen®, France).

For Ultra High Vacuum (UHV) analyses (Scanning Electron Microscopy and ToF-SIMS), sessile cells were fixed using the following procedure: after postfixation for 1 h in 3% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.2) at 4 °C, the surface was rinsed with the buffer (NaCl 0.15 M). Adhering cells were then dehydrated using a graded ethanol series (30%, 50%, 70%, 90%, and 100% two times for 15 min each) and subjected to acetone dehydration.

The fixation and dehydration treatments were also given to the reference samples.

Epifluorescence and FE-SEM images characteristic of stainless steel surfaces with bacteria (treated samples) show that in the early stage of biofilm formation (1 h) the surface of the stainless steel substrate is partially covered by *E. coli* microorganisms: about 20% of the surface is covered by adherent bacteria as determined by epifluorescence microscopy (at least 10 fields of view were counted in a representative experiment) (Fig. 1).

2.4. FE-SEM measurements

FE-SEM analyses were obtained using a Jeol 7000F microscope. The stainless steel coupons were sputter-coated with Au/Pd before analysis.

2.5. ToF-SIMS measurements

ToF-SIMS analyses were obtained using a ToF-SIMS 5 spectrometer (IonTof – Munster Germany). The spectrometer was operated at a pressure of 10^{-9} mbar. A pulsed 25 keV Bi^+ primary ion source was employed for analysis, delivering 1.0 pA over a 100 μm × 100 μm area. This set up allowed us to obtain detailed elemental and molecular information from the analyzed sample surface. Depth profiling was done using a 1 keV Cs^+ sputter beam with a 45° incidence to the specimen surface giving a 50 nA target current over a 300 μm × 300 μm area. Negative ion depth profiles were recorded because these have a better sensitivity for oxides. Data acquisition and post-processing analyses were performed using the Ion-Spec software.

To determine the evolution of the surface composition with sputter time (i.e. with depth), the following ions have been selected: (i) C^- (mass: 12) that represents both carbon contamination on the extreme surface and the bacteria deposited on the surface of the stainless steel sample, (ii) OH^- (mass: 17) and $^{18}O^-$ (mass: 18) that are characteristic signals of an oxidized layer (oxide and/or hydroxide). The $^{18}O^-$ isotope signal was used to analyse the oxygen content because the $^{16}O^-$ (mass: 16) signal often saturates the detector, (iii) FeO_2^- (mass: 88), NiO_2^- (mass: 91), CrO_2^- (mass: 84) and MoO_2^- (mass: 128) that are characteristic signals for Fe, Ni, Cr and Mo coming from oxides and/or hydroxides, (iv) Fe_2^- (mass: 112), Ni_2^- (mass: 118) and Cr_2^- (mass: 104) that are characteristic signals for Fe, Ni and Cr coming from a metallic substrate. The Mo_2^- (mass: 192) signal is not used as it is hardly observed in negative polarity due to a too low sensitivity, and (v) CH_2^- (mass: 14), $^{13}CN^-$ (mass: 27), P^- (mass: 31), S^-(mass: 32), PO^- (mass: 47), SO^- (mass: 48) that are characteristic signals for the organic matter (bacteria and/or biomolecules).

3. Results and discussion

It is well known from previous study that a bare stainless steel substrate (without bacteria) is covered by a native oxide film with a thickness of ~2–3 nm [19,20]. A schematic representation of a passivated surface is shown in Fig. 2a. From a theoretical point of view, a ToF-SIMS profile of such system should exhibit (i) a first region characterized by a high intensity oxide signal (from the passive oxide film) and (ii) a second region, characterized by an intense metallic signal (from the metallic substrate). In agreement with this theoretical profile, the experimental profile obtained on stainless steel without bacteria (reference sample) (Fig. 2b) can be divided into two regions corresponding to (i) the passive oxide

Table 1
Chemical composition of the stainless steel used in this study (in wt% or ppm).

Element	Ni (wt%)	Cr (wt%)	Mo (wt%)	Fe (wt%)	Mn (wt%)	P (wt%)	Si (wt%)	Cu (wt%)
	10.2	16.77	2	Bal.	⩽2	⩽0.045	⩽1	0.32

Element	Ti (wt%)	Co (wt%)	Sn (wt%)	C (ppm)	S (ppm)	N (ppm)	O2 (ppm)
	⩽0.001	0.13	0.013	⩽300	⩽150	⩽1100	35