



SULPHATE REDUCING BACTERIA IN BIOFILMS ON THERMOSETTING POLYMERS/Zn COMPOSITE LAYERS

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ABSTRACT

Bacterial adhesion to surfaces is the first step in the formation of a biofilm and has been studied extensively over the past decades in many diverse applications. Sulphate Reducing Bacteria (SRB) is a group of phylogenetically diverse anaerobic microorganisms that were first discovered by Beijerinck in 1895. This work investigates the attachment of Sulphate Reducing Bacteria and the modification of roughness before and after the attachments on the surfaces of zinc and thermosetting polymers/zinc composite layers obtained by electro co-deposition. There were used two types of thermosetting polymers: phenol – formaldehyde resin (type NOVOLAC) and epoxy resin. For investigations of the surfaces were used atomic force and epifluorescence microscopy methods (AFM and EFM, respectively). Sessile bacteria on coupons were stained with 4', 6-diamidino-2-phenylindol (DAPI) and visualized by EFM as well as AFM. The best imaging conditions for AFM were assessed.

KEYWORDS: biofilm, composite layers, Sulphate Reducing Bacteria, Atomic Force Microscopy, Epifluorescence Microscopy, roughness

1. Introduction

Bacterial adhesion mechanism is complex and many factors affect cell adhesion [1]. Some bacteria have been reported to accelerate the corrosion of metals, while others influence the corrosion behavior in a beneficial way. *Desulfovibrio desulfuricans*, *Pseudomonas* sp. and *Bacillus* sp. can accelerate corrosion. On the other hand, *Bacillus subtilis* has been shown to inhibit the corrosion of aluminum 2024 by secreting polyglutamate and polyaspartate [2, 3], while *Pseudomonas flava* inhibits corrosion by forming a phosphate film [4].

Central to the phenomenon of microbially induced corrosion is the formation of biofilm on the metal surface. The biofilms are formed by microbial aggregates and extracellular polymeric substances (EPS). The EPS creates a microenvironment for sessile bacteria and allow for the development of synergistic relationship. Their main components are not only polysaccharides, but also proteins, lipids and nucleic acids in minor proportion [5].

Sulphate Reducing Bacteria (SRB) is a group of phylogenetically diverse anaerobic microorganisms that were first discovered by Beijerinck, in 1895. At

present, 14 genera have been identified, the two most established genera of SRB being *Desulfovibrio* and *Desulfotomaculum* [6, 7]. The biofilms are involved in both beneficial and detrimental effects: one beneficial aspect is their potential use as biosurfactants in tertiary oil production and their capacity to trap heavy metals; as detrimental effect, biofouling increases friction resistance and produces changes in metallic surface properties (hydrophobicity, roughness, color, etc.); finally, biofilms participate in biocorrosion by bind with metal ions [8].

The atomic force microscope is a mechanical imaging device that requires minimal sample preparation and creates three-dimensional images with high spatial resolution. By combining AFM and EFM, two techniques with complementary strengths and weaknesses are joined to yield a powerful tool for the investigation of biological samples [9 - 12]. Using this methods, it was reported the attachment of SRB on different type of materials: 316 stainless steel [13 - 14], D36 carbon steel [15], C1018 carbon steel [16], alloy 625 and austenitic stainless steel [17], Q235 steel [18], heat resistant steel 1Cr18Ni9Ti [19], ASTM grade 2 titanium [20], cerium-doped TiO₂ film

on 304 stainless steel [21], polyurethane foam Ringlace® and lava rock [22], polyurethane foam (PU), vegetal carbon (VC), low-density polyethylene (PE) and alumina-based ceramics (CE) [23].

The literature is penurious referring to attachment of SRB on metal and especially on composite layers. For this reason, in the present study the work was focus on performing AFM coupled with EFM studies in order to observe the influence of materials structure (pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers prepared by electro co-deposition) on SRB attachment.

2. Materials and methods

2.1 Substratum and biofilm formation

Three types of surfaces were prepared by electro co - deposition: pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers. They were electrochemically deposited from a bath with the following composition: 310g/L ZnSO₄ · 7H₂O; 75g/L Na₂SO₄ · 10H₂O; 30g/L Al₂(SO₄)₃ · 18H₂O. The pH of the solution was 3.8.

These layers were electrodeposited on DC04 steel as substrate. Suspension for electro co - deposition of composite layers was prepared by adding phenol formaldehyde resin particles, respectively epoxi resin particles (mean diameter 6 – 10µm) to the solution to give a concentration of 10g/L in the zinc electrolyte plating bath. Electro co - deposition took place in the bath at a temperature of 25^oC, current density of 4A/dm², time for electrodeposition 30min. The suspension bath was stirred by a mechanical stirrer at a constant rotational speed of 800rpm.

In this investigation, SRB from the University of Duisburg Essen Biofilm Centre, Aquatic Biotechnology were used for bacterial adhesion tests. The pH of solution with cells suspension was 6.2. The attachment of cells was made in the following steps: putting a drop from the prepared solution cells on the tested surfaces; waiting to dry (15-20min); next, coupons were incubated in bacterial suspension of SRB (1 ppb organic matter 10⁹cells/mL) for 24h to allow for the attachment and biofilm formation with 2, 5% glutaraldehyde.

2.2. Instrumentation

Biofilm and attached cells on pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers were investigated with combined AFM and EFM methods. Subsequently, they were stained with 0.01% (wt/vol) DAPI for 10min and visualized at the epifluorescence microscope.

A NanoWizardII atomic force microscope (JPK Instruments, Germany) and an upright epifluorescence microscope (AxioImager A1m; Zeiss,

Germany) were combined using the BioMaterialWorkstation (JPK Instruments). Throughout the present study the prototype of this new system was used. The key feature of the BioMaterialWorkstation was a shuttle stage that carried the actual sample precisely fixed on a glass slide. This shuttle stage could be transferred between the atomic force microscope and the epifluorescence microscope, giving a precise positioning of the stage on both microscopes.

For AFM images, silicon cantilever CSC37 A (Mikromasch, Estonia) with the following features was used: typical length, 250 µm; width, 35 µm; thickness, 2 µm; resonance frequency, 41 kHz; and nominal force/spring constant, 0.65 N/m. Each AFM image consists of 512 by 512 pixels. AFM images were performed by contact mode in air.

3. Results and discussion

The surface structure of pure zinc layer and thermosetting polymers/Zn composite layers under atomic force microscope are presented in Figs. 1 – 3. It can be observed that the surface of zinc is made up of regular crystals.

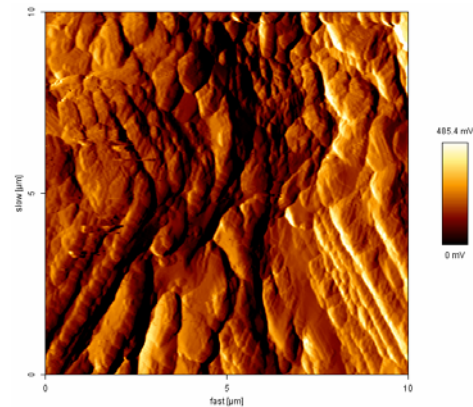


Fig. 1. 2D - AFM image of untreated pure zinc surface

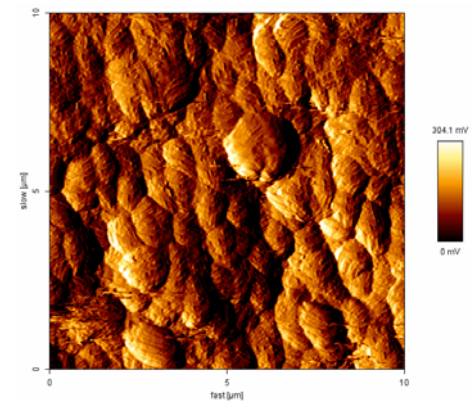


Fig. 2. 2D - AFM image of untreated surface PF resin/Zn composite layer

The thermosetting polymers particles co - deposited with zinc disorder the regular crystal structure and the structure of the zinc matrix becomes finely crystalline.

The pure zinc layers have a rather regular surface, whereas the composite layer surfaces have finer grains structure with particles of resin uniform by distributed on the surfaces. The thermosetting polymer could have an inhibition effect of zinc crystals growth and a catalytic effect in increasing nucleation sites.

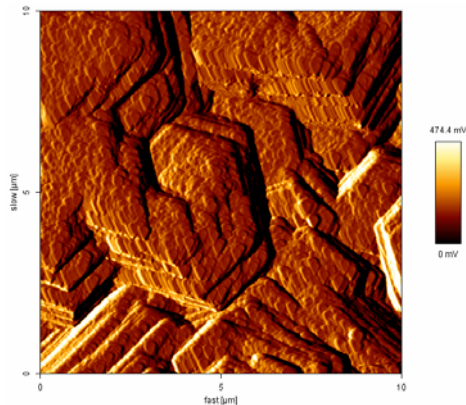


Fig. 3. 2D - AFM image of untreated surface epoxy resin/Zn composite layer

Epifluorescence microscopy (EFM) images of a DAPI – stained biofilm sample of SRB on the surface of zinc and thermosetting polymers/Zn composite layers are presented in Figs. 4 – 6.

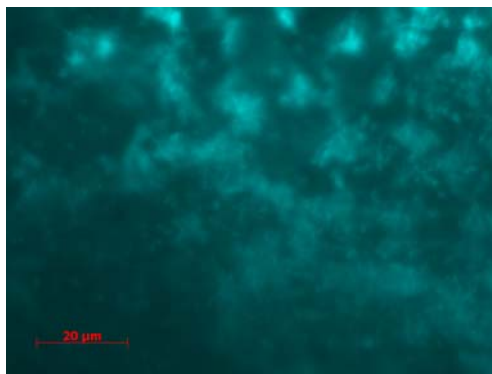


Fig. 4. EFM image of the SRB attachment and EPS formed on pure zinc surface

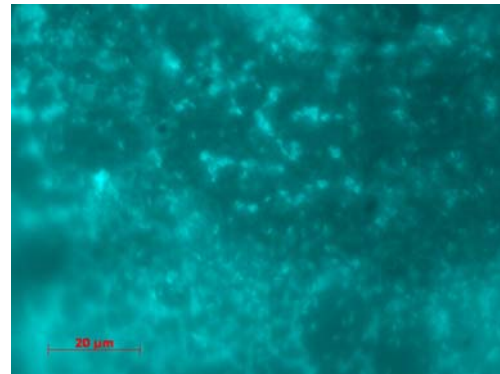


Fig.5. EFM image of the SRB attachment and EPS formed on PF resin/Zn composite layers surface

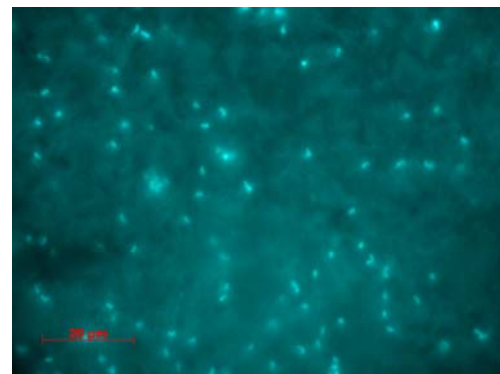


Fig.6. EFM image of the SRB attachment and EPS formed on epoxy resin/Zn composite layers surface

From the EFM images, it was observed that the attachment of SRB on thermosetting polymers/Zn composite layers surface is lesser than on pure zinc surface. Those facts indicated that the thermosetting polymers/Zn composite layers are more resistant to the attack of microorganisms like SRB.

Figs. 7 – 9 show the 3D images of the AFM scan acquired by contact mode in air on pure zinc layers and thermosetting polymers/Zn composite surfaces untreated and after SRB attachment with biofilm and EPS formation.

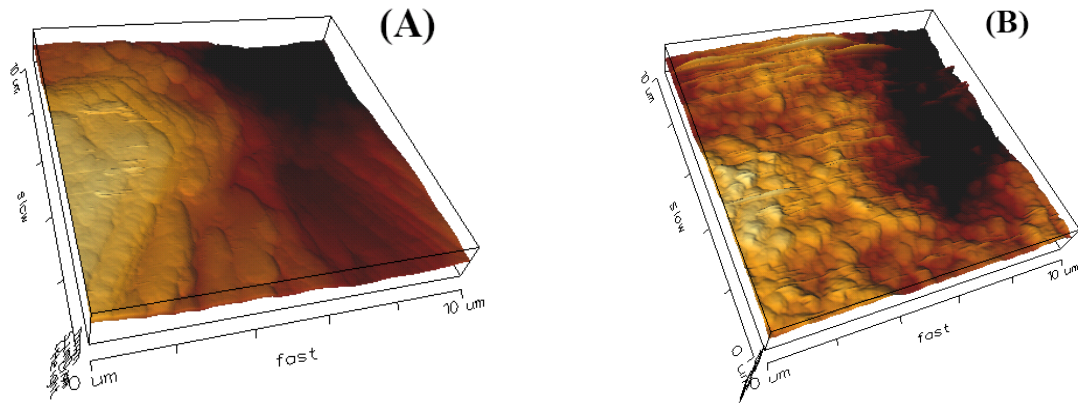


Fig. 7. 3D - AFM images of pure zinc surface: (A) - untreated; (B) - with SRB bacteria

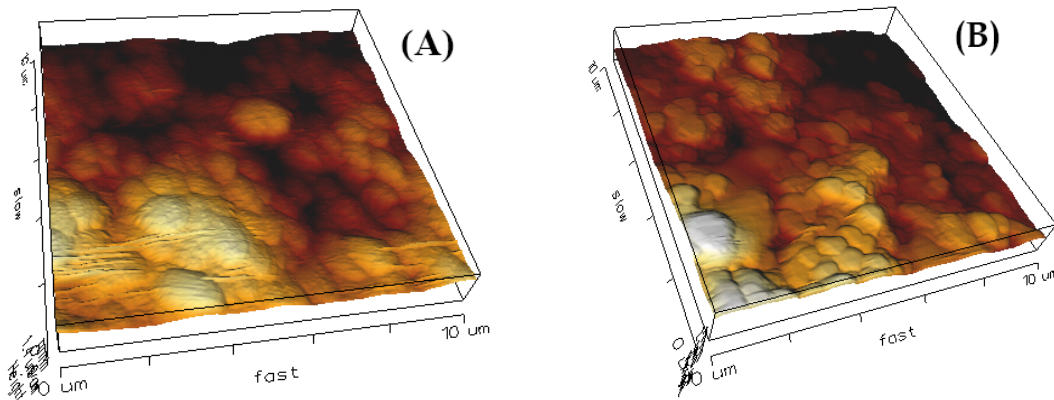


Fig. 8. 3D - AFM images of PF resin/Zn composite layer surface (A) - untreated; (B) - with SRB bacteria

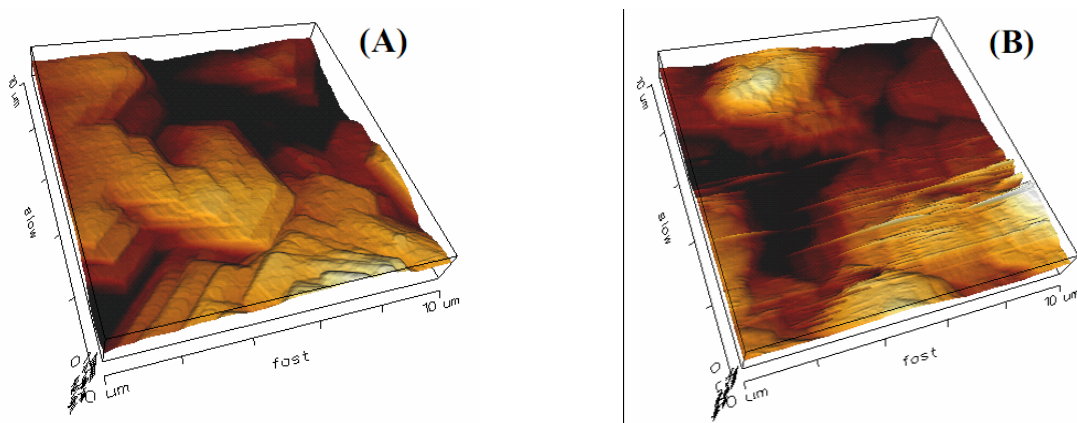


Fig. 9. 3D - AFM images of epoxy resin/Zn composite layer surface (A) - untreated; (B) - with SRB bacteria

The differences between untreated surfaces and treated with SRB are visible, representing the attached cells of Sulfate Reducing Bacteria on the surfaces, biofilm and EPS formation. EFM – AFM images indicate an adherence process of the microorganisms on the tested surfaces. The use of microscopy to count adhered cells on surfaces is a viable technique, since,

on a microscopic scale, surfaces can be found to have cracks and crevices, quite unlike the macroscopic appearance. These surface imperfections protect the microorganisms against removal by swab or rinse. The histograms of the scanned surfaces before and after the attachment of SRB are presented in Fig.10 - 12.

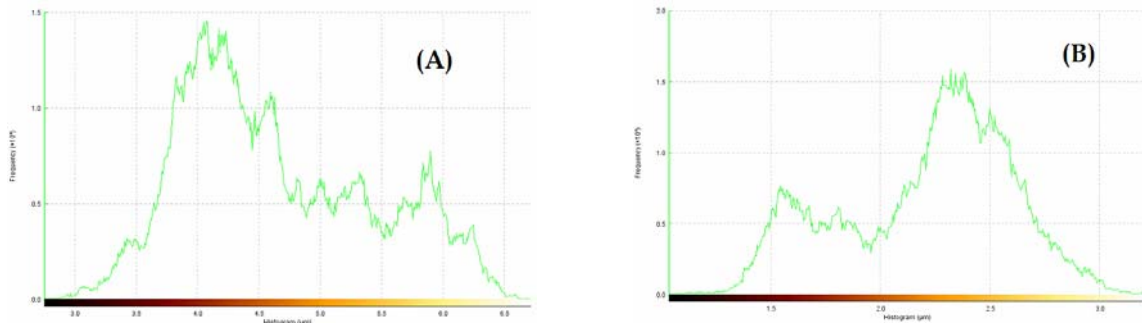


Fig. 10. Histograms of the scanned surfaces for pure zinc before (A) and after the SRB attachment (B)

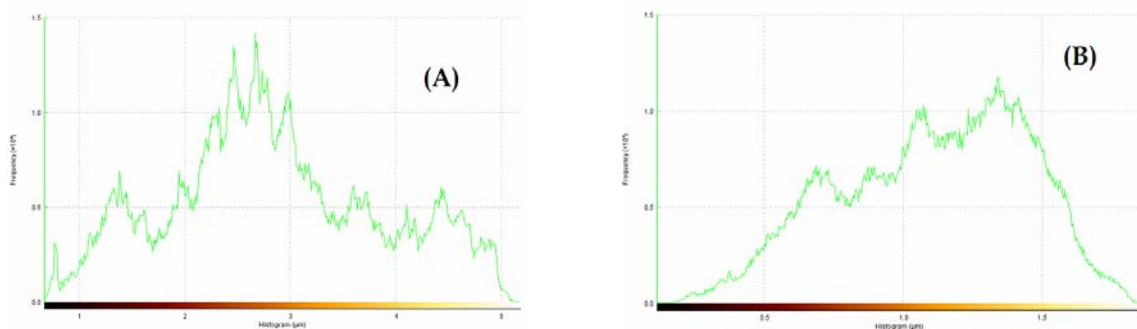


Fig. 11. Histograms of the scanned surfaces for PF resin/Zn composite layers before (A) and after the SRB attachment (B)

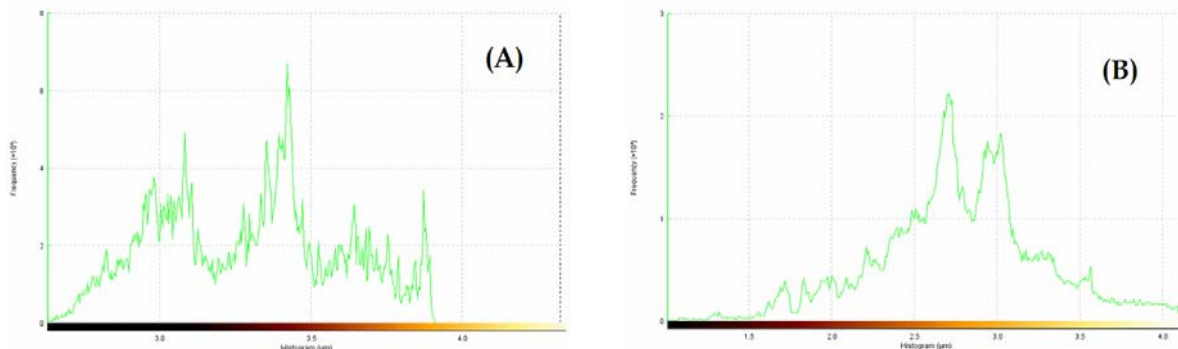


Fig. 12. Histograms of the scanned surfaces for epoxy resin/Zn composite layers before (A) and after the SRB attachment (B)

These histograms were used to calculate the roughness of the tested surfaces before and after the attachment of SRB, biofilm and EPS formation.

Roughness is a measure of the texture of a surface and plays an important role in determining how a real system will interact with the environment.

It is quantified by the vertical deviations of a real surface from its ideal form. If these deviations are large, the surface is rough; if they are small the surface is smooth.

The variation of the surfaces roughness for pure zinc and thermosetting polymers/Zn composite layers before and after attachment of SRB is shown in Fig. 13.

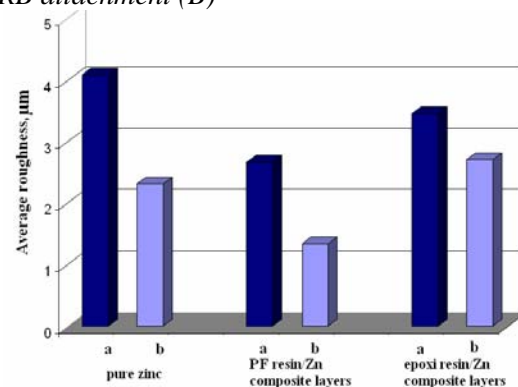


Fig.13. Variation of roughness surfaces of pure zinc and thermosetting polymers/Zn composite layers before (a) and after the attachment of SRB (b)



It could be observed that the values of roughness for composite layers without bacteria are smaller than the roughness of pure zinc.

The thermosetting polymer type PF resin and epoxy resin act as reducing the crystals size of electrodeposited zinc during co-deposition. For all systems tested the surfaces roughness decreases after the attachments of bacteria. That could indicate an increase of the uniformity for all tested surfaces after the attachments of SRB, biofilm and EPS formation.

From the EFM images and the values of the roughness it was observed that the attachment of SRB on thermosetting polymers/Zn composite layers is more reduced than on pure zinc surface. For pure zinc surfaces, the difference between the value of roughness for untreated and surface with SRB is bigger than other tested surfaces. That indicates a lot of bacteria attached on this surface, creating biofilm, EPS and corrosion product conducting to a smooth surface. Those facts indicated that the thermosetting polymers/Zn composite layers are more resistant to the attack of microorganisms like SRB.

4. Conclusions

The Sulfate Reducing Bacteria were attached on the pure zinc and thermosetting polymers/Zn composite layers.

Bacterial attachment on the surfaces is a complicated process that is affected by material surface (pure metal or composite layers).

From the epifluorescence microscopy and atomic force microscopy images it could be observed that the thermosetting polymers/Zn composite layers are more resistant to the attack of the Sulphate Reducing Bacteria than pure zinc layers.

The surface roughness decreases after the attachments of bacteria, biofilm and EPS formation.

The new system for combining imaging of AFM and EFM on pure zinc and thermosetting polymers/Zn composite layers is feasible for the application to study the biofilm formation by Sulfate Reducing Bacteria on these surfaces.

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