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Corrosion behaviour of titanium in the presence of *Streptococcus mutans*

Júlio C.M. Souza [a,b,*], *Pierre Ponthiaux* [c], *Mariana Henriques* [d], *Rosário Oliveira* [d], *Wim Teughels* [e], *Jean-Pierre Celis* [f], *Luís A. Rocha* [a]

[a] *Centre for Mechanical and Materials Technologies, CT2M, Universidade do Minho, P-4800-058 Guimarães, Portugal*
[b] *Dental School, School of Health Sciences (FCS), Universidade Fernando Pessoa (UFP), Porto P-4200-250, Portugal*
[c] *Ecole Centrale Paris, Chemical Engineering Laboratory (LGPM), F-92290 Châtenay-Malabry, France*
[d] *IBB – Institute for Biotechnology and Bioengineering, Universidade do Minho, P-4710-057 Braga, Portugal*
[e] *Department of Periodontology, KU Leuven, Leuven B-3000, Belgium*
[f] *Department of MTM, KU Leuven, Leuven B-3001, Belgium*

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ABSTRACT

Objective: The main aim of this in vitro study was to evaluate the influence of *Streptococcus mutans* on the corrosion of titanium.
Methods: *S. mutans* biofilms were formed on commercially pure titanium (CP-Ti) square samples (10 mm × 10 mm × 1 mm) using a culture medium enriched with sucrose. Open circuit potential (OCP) and electrochemical impedance spectroscopy (EIS) measurements were used to evaluate the corrosion behaviour of CP-Ti in the presence of *S. mutans* in Fusayama's artificial saliva. The corrosion of biofilm-free CP-Ti samples was also evaluated in artificial saliva. Biofilms biomass was measured by spectrophotometry, using crystal violet staining, after 1, 2 and 7 days.
Results: The OCP values recorded on CP-Ti in the presence of *S. mutans* (-0.3 ± 0.02 V vs. SCE) was lower than those on biofilm-free CP-Ti (-0.1 ± 0.01 V vs. SCE) after 2 h of immersion in artificial saliva ($p < 0.05$). That reveals a high reactivity of titanium in presence of *S. mutans*. Impedance spectra revealed the formation of a compact passive film on titanium in artificial saliva or in the presence of a 2 days old *S. mutans* biofilm even though the corrosion resistance of CP-Ti has decreased in presence of a *S. mutans* biofilm.
Conclusion: The presence of bacterial colonies, such as *S. mutans*, negatively affected the corrosion resistance of the titanium.

1. Introduction

A biofilm consists of a well-organized community of microbial cells, including one or multi-species agglomerates, surrounded by an extracellular matrix composed of polysaccharides, nucleic acids, H_2O, proteins and other substances.[1–3] Biofilm accumulation is an important factor that can cause failures of oral rehabilitation systems, especially considering the pathogenic potential of some bacteria such as *Streptococcus mutans*, *Porphyromonas gingivalis* and *Prevotela intermedia* which promote dental caries or periodontal diseases.[1–6] Since specific types of acid-producing bacteria can promote the degradation of hard

* Corresponding author at: Universidade do Minho, Centre for Mechanical and Materials Technologies (CT2M), Departamento de Engenharia Mecânica, Campus Azurém, P-4800-058 Guimarães, Portugal. Tel.: +351 253 510231; fax: +351 253 516007.
E-mail addresses: lrocha@dem.uminho.pt, jsouza@dem.uminho.pt (Júlio C.M. Souza).
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tooth structures, restorative materials, such as dental composites or dental amalgam, can also be deteriorated during a biocorrosion process.[1,8–13] Among the several microorganisms present in the oral cavity, *Streptococcus mutans* is one of the utmost important due to its capacity to release lactic acid and to grow in acidic environments becoming a powerful corrosive microorganism.[1,13–15] Moreover, it grows in both aerobic and anaerobic environments, and can be found at different habitats in the oral cavity.[1,3] In fact, oral biofilms with a high proportion of *S. mutans* cause a pH decrease in the oral cavity promoting the demineralization of enamel, dentine, and cementum as well as the corrosion of dental restorative materials.[1,8–13] However, the corrosive role of *S. mutans* depends on the sucrose concentration present on its environment and in its adhesion to the oral surfaces.[1,14–17] Although *S. mutans* is not directly responsible for periodontal inflammations, it is known that oral biofilms consist in consortia of other species depending on environmental conditions like oxygen, nutrients, and pH.[1,17–22] In addition, the biofilm structure can pick up external acidic substances from dietary, as well as acidic substances produced from microbial metabolism.[1,23,31]

In dentistry, commercially pure titanium is the first choice for dental implants, while titanium alloys (e.g. Ti6Al4V and Ti15Zr4Nb4Ta) are desirable for removable and fixed dental prostheses, due to their good corrosion resistance, low density, high mechanical strength and biocompatibility.[24–28] Indeed, titanium is a material with a high corrosion resistance compared to other metallic materials used in oral rehabilitation thanks to a compact titanium oxide (TiO_2) film at its surface in oxygen containing environments.[9–12,29–31] However, the dissolution of the TiO_2 film may occur in certain media such as those containing high fluoride concentrations, hydrogen peroxide (H_2O_2), and lactic acid, like it can occur in the oral cavity.[7,8] Moreover, the corrosion of titanium increases when F^-, H_2O_2, and lactic acid are combined, as revealed by Mabilleau et al.[8] Corrosion of titanium results in the release of metallic ions into the surrounding tissues that can stimulate an initial inflammatory response, and a consequent toxic, mutagenic and/or carcinogenic reaction.[32,33] If severe, the effect of corrosion may be visible in vivo resulting in a change of surface colouration or perimplant inflammations due to the released ions.[4,8] Guindy et al.[7] reported the failure of six dental implant systems caused by corrosion of the metallic suprastructure. In that study, areas with clear signs of localized corrosion on implants and inner crown surfaces were detected by light and scanning electron microscopy on all six implants and inner crown surfaces. Corrosion causes a material loss that leads to a dimensional misfit between prosthetic crown and abutment or between abutment and implant.[32,33]

Considering the increased use of titanium in oral rehabilitation, corrosion studies of titanium and its alloys in the presence of microorganisms become very important due to an enormous number of microorganisms and corrosive substances present in the oral cavity which vary from patient to patient and on the oral environmental conditions. The study of the corrosion resistance of titanium in the presence of microorganisms can determine the performance of implant-supported prostheses. As a result, the reduction of restorative material loss by corrosion phenomena can increase the long-term success of dental implant systems. The main goal of this work was to evaluate the influence of *S. mutans* biofilms typically present in the oral cavity on the corrosion of titanium, through electrochemical techniques.

2. Materials and methods

2.1. Preparation of samples and fluoridated artificial saliva solutions

Square samples (10 mm × 10 mm × 1 mm) were cut from sheets of commercially pure titanium (CP-Ti) (VSMPO TIRUS, US, ASTM B 348, Cp-Ti Grade 2). The samples were wet ground on SiC abrasive papers (Struers A/S, Denmark) down to 1200 Mesh. After grinding, samples were first cleaned in isopropyl alcohol (Sigma–Aldrich, USA) for 10 min and then in distilled water for 5 min using an ultrasonic bath. These samples were stored in a desiccator for 1 day, and sterilized before biofilm formation or electrochemical measurements by autoclaving at 121 °C for 15 min.

A modified Fusayama's artificial saliva formulation[34] (Table 1) was used as stock solution in this in vitro corrosion study. The electrochemical behaviour of metallic materials in that solution has been reported to be similar as in human saliva.[34]

2.2. Bacterial strains and growth conditions

S. mutans ATCC 25175 were microaerophilically grown for 48 h at 37 °C in agar plates with 32 g/L of BHI agar (Bacto, Difco, USA) supplemented with 3 g/L of yeast extract and 200 g/L of sucrose (Bacto, Difco, USA). The bacterial cells were inoculated in Tryptic Soy Broth (TSB, Bacto, Difco, USA) supplemented with 3 g/L of yeast extract and 200 g/L of sucrose for 18 h at 37 °C and 150 rpm. After incubation, cells were harvested by centrifugation for 10 min at 4 °C and 5000 rpm and washed twice with Phosphate Buffer Solution (PBS, Sigma–Aldrich, USA). Then, the cells were re-suspended in TSB supplemented with mucin (2.5 g/L), peptone (5 g/L), urea (1 g/L), yeast extract (2 g/L) and sucrose (200 g/L) to obtain an optical density (OD) of about 0.6 at A_{630}, corresponding to approximately 1×10^8 CFU/ml.[5,20–22] The OD at 630 nm was measured using a spectrophotometer (BioTek, USA). This cell suspension was the inoculum for biofilm formation assays.

2.3. Biofilm formation and analysis

Titanium samples were placed into 24 well-plates, each containing 2 ml of *S. mutans* inoculum (1×10^8 CFU/ml) and

Table 1 – Composition of the stock Fusayama's artificial saliva solution used in this work.

Compounds	(g/l)
NaCl	0.4
KCl	0.4
$CaCl_2{\cdot}2H_2O$	0.795
$Na_2S{\cdot}9H_2O$	0.005
$NaH_2PO_4{\cdot}2H_2O$	0.69
Urea	1

incubated at 37 °C. After 24, 48 and 168 h (7 days) of incubation, the samples were transferred for new well-plates and washed twice with PBS for the evaluation of biomass by the crystal violet (CV) staining method.[5,30,31] Those time points were selected in order to be possible to evaluate the initial phases of biofilm formation immediately after the insertion of titanium-based abutments or prosthetic frameworks in the oral cavity up to 48 h. The other time point was selected as a longer one in order to evaluate the increase of the biofilm density and pH. The pH of the culture medium containing *S. mutans* was measured every day using a pH metre (Mettler Toledo 340, Brazil). Then, the titanium samples were immersed in 1 ml of methanol for 15 min to allow cell fixation. After that, methanol was removed and the titanium samples were dried at room temperature and 1 ml CV (1%) was added to stain the bacterial biofilm for 5 min. After that, the titanium samples were dip-washed in distilled water, dried at room temperature, and transferred to new 24-well plates containing 1 ml of acetic acid (33%) in order to remove the CV solution from cells. The suspension was aspirated (aliquots of 200 μl) and placed in 96-well plates to determine the OD at 540 nm.

For microscopic analyses, surfaces covered with biofilms were washed two times in PBS and fixed in glutaraldehyde 2% for 5 min. Then surfaces were washed three times in PBS, and dehydrated through a series of graded ethanol solutions (50, 70, 80, 90, 100%). Samples covered with *S. mutans* biofilms were sputter-coated with gold, and analyzed by Scanning Electron Microscopy (SEM, S360 LEICA CAMBRIDGE) at 15 kV and by Field-Emission Scanning Electron Microscopy (FESEM, FEI QUANTA 400 FEG) at 5–10 kV at an angle of 60°.

2.4. Corrosion measurements

Samples covered or not with *S. mutans* biofilms were mounted in an acrylic electrochemical cell connected to the external electrical wiring. The electrochemical tests were carried out with a Voltalab PGZ100 potentiostat (Radiometer Analytical) coupled to the Voltamaster 4 software used for electrochemical control and data analyses. The open circuit potential (OCP) is defined as the potential of an electron conductive material immersed in an ion conductive electrolyte and measured against a reference electrode. In this work, a standard calomel reference electrode (SCE, Radiometer Analytical, XR110 model) was used. A Pt-electrode (Radiometer Analytical, M231PT model) was used as counter electrode in impedance and potentiodynamic polarization tests. The test samples were connected as working electrode. Since on immersion of a test sample in the Fusayama's artificial saliva, the OCP evolves with time, a waiting time was inserted till the OCP stabilized. The titanium test samples covered with biofilms were tested in Fusayama's artificial saliva solution A group of titanium samples without biofilms used as control group, was immersed in sterilized growth medium for 48 h before their OCP was measured in Fusayama's solution.

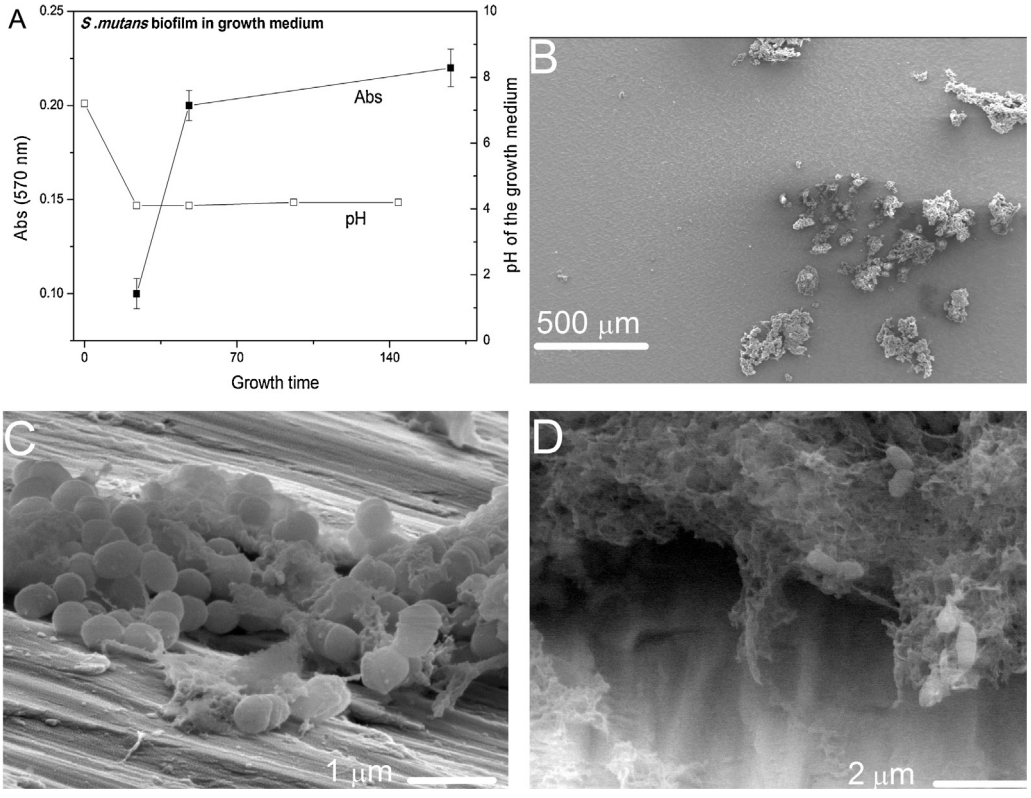

Fig. 1 – Crystal violet absorbance (Abs) of *S. mutans* biofilm biomass formed on titanium surfaces and pH of biofilm medium after 24, 48, and 168 h (7 days) of growth (growth in TSBMPY20%S, 37 °C, 150 rpm). Images of *S. mutans* biofilms formed (grown in TSBMPY20%S, 37 °C, 150 rpm) on titanium surfaces after (C) 24, and (B and D) 48 h. Images obtained by FEGSEM operated in secondary electrons (SE) mode at 10 kV and an angle of 60°.

2.5. Statistical analysis

The results were statistically analyzed via one-way analysis of variance (ANOVA), at a significance level of $p < 0.05$ by using the SPSS 17.0 software for Windows (Chicago, IL, USA).

3. Results

3.1. Biofilm analysis

The biomass of *S. mutans* biofilms formed on titanium samples was determined after 24, 48, and 168 h by absorbance measurements after CV staining (Fig. 1). A significant increase ($p < 0.05$) of biomass occurs after 48 h of incubation. However, no statistically significant differences ($p < 0.05$) were found between the biomass present after 48 and 168 h (7 days) of incubation. Also, as shown in Fig. 1A, the pH of the growth medium becomes acidic (pH ca. 4) during the growth of the biofilm. The morphology of *S. mutans* biofilms formed on titanium surfaces is shown in Fig. 1B–D. A higher biofilm accumulation is noticed after 48 h of growth than after 24 h. Additionally, a higher production of extracellular polysaccharides and the existence of canals below and inside a biofilm grown for 48 h, are observed (Fig. 1). SEM images did not reveal the presence of a localized corrosion on the titanium surfaces after a biofilm growth for 48 h.

3.2. Corrosion measurements in artificial saliva solutions in the presence of S. mutans biofilms

A decrease of the OCP was recorded on titanium covered with *S. mutans* biofilms grown for 48 h as shown in Fig. 2. After an immersion for 2 days in a sterile growth medium, the OCP values recorded on titanium samples without biofilms evolve towards more noble values (0 V vs. SCE) in artificial saliva.

Also, EIS tests were performed for 48 h to evaluate the state of the titanium passive film in the presence of biofilms (Fig. 3A). The EIS spectra (Fig. 3A) for titanium surfaces free of biofilms reveal values of the phase angle approach from −90° and a higher inclination of the slopes ([Z] vs. Frequency) than

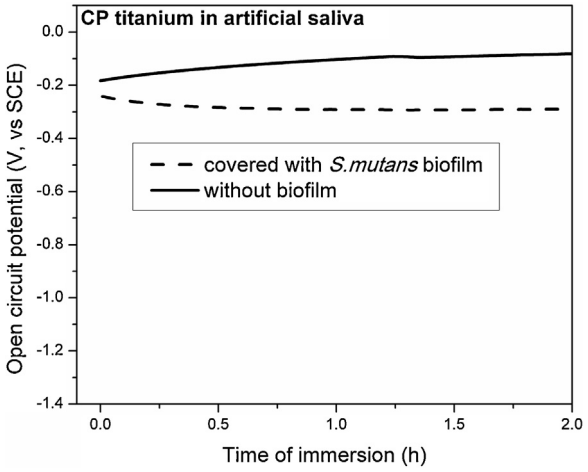

Fig. 2 – Evolution of open circuit potential (OCP) recorded on titanium covered or not with *S. mutans* biofilms grown during 48 h in TSBMPY20%S (37 °C, 150 rpm), and immersed in Fusayama's artificial saliva.

those recorded on titanium covered with *S. mutans*. That indicates higher values of the total impedance for titanium without biofilms than in presence of biofilms ($p < 0.05$).

An equivalent circuit was derived from non-linear square fitting of EIS spectra, as shown in Fig. 3B. That circuit known as Randle's circuit consists of a passive film capacitance (C_f) in parallel with a polarization resistance of the passive film (Rp_f) in series with a solution resistance (R_s). Randle's circuit indicates a capacitive behaviour of titanium surface in presence of a compact titanium oxide film in both cases (Fig. 3B). In other words, there was no formation of defects such as pits on the titanium surfaces with and without biofilms.

The values of Rp_f and C_f obtained by fitting of EIS spectra are shown in Fig. 4. The equivalent electrical circuits as well as experimental and theoretical values showed an adequate fitting in agreement to chi-square values (χ^2) between 10^{-4} and 10^{-5}. After analyzes of C_f and Rp_f values by ANOVA, significant

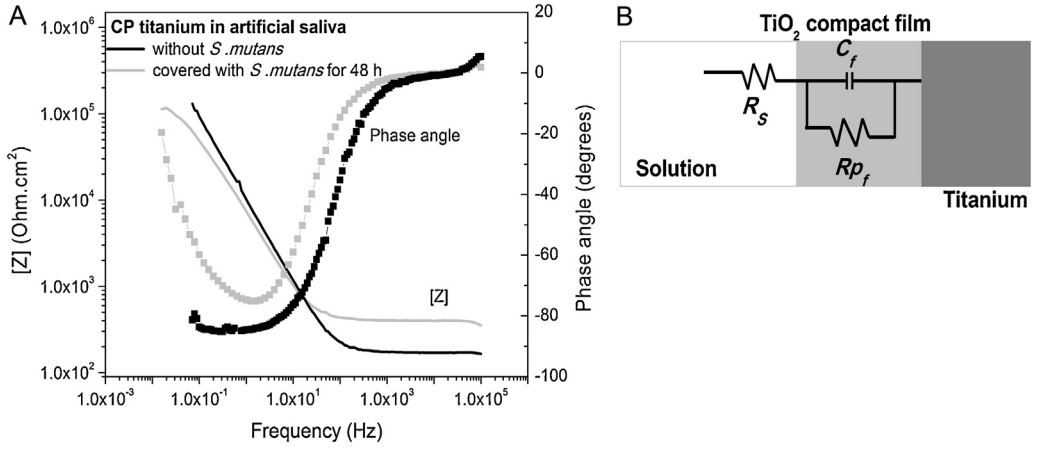

Fig. 3 – (A) EIS spectra (bode representation) for titanium covered or not with *S. mutans* biofilms (48 h of growth in TSBMPY20%S, 37 °C, 150 rpm) and (B) the corresponding electrical circuit.

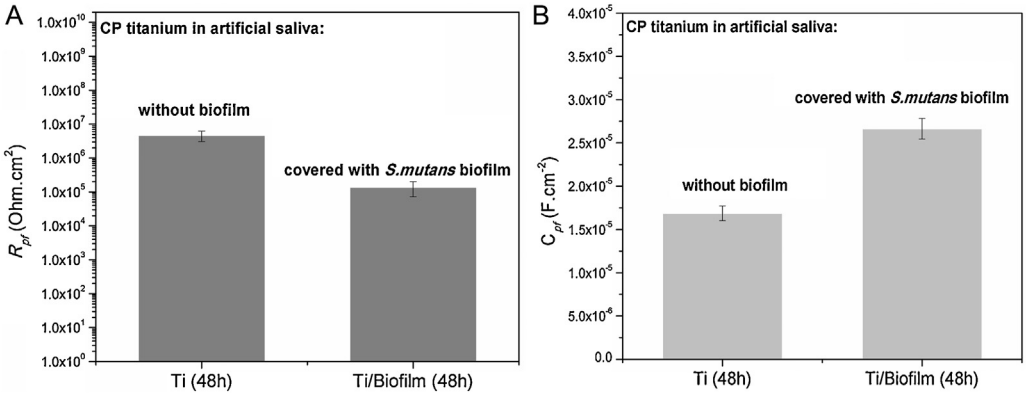

Fig. 4 – (A) Polarization resistance (Rp_f) and (B) capacitance of titanium passive film (C_f) with and without *S. mutans* biofilms grown for 48 h in TSBMPY20%S (37 °C, 150 rpm) when immersed in artificial saliva.

differences ($p < 0.05$) were found between the two groups of titanium samples. The lowest values of Rp_f (Fig. 4A) were observed for titanium covered with biofilms what confirms the decreased corrosion resistance in presence of *S. mutans*. As shown in Fig. 4B, the values of C_f for titanium without biofilms are lower than those for titanium covered with biofilms, although in both cases the impedance results indicate the presence of a passive film. Additionally, these values of C_f suggest a higher thickness of the TiO_2-film in absence of biofilms compared to titanium covered with biofilms.

4. Discussion

In this work, the corrosive effect *S. mutans* on titanium was evaluated by electrochemical techniques associated to biofilm density and microscopic analyses. *S. mutans* agglomerates surrounded by their extracellular matrix could be detected on titanium over a period of 48 h of growth (Fig. 1). Considering that *S. mutans* agglomeration, the colonization of different kinds of surfaces and materials by *S. mutans* has been investigated in previous studies.[1,6–8] *S. mutans* has been classified as hydrophobic performing its initial adhesion on titanium surfaces supported by glycoprotein as e.g., mucin or polysaccharides extracellular matrix.[1,16–18] Electrostatic interactions on the adsorption of mucin to titanium as well as between mucin and *S. mutans*, are responsible for the initial adhesion of *S. mutans* cells[1,16–18] (Fig. 1B and C). Also, *S. mutans* growth can be enhanced by high sucrose concentration, so that the production of extracellular matrix leads to biofilm agglomeration[1,16–19] (Fig. 1D). The stabilization of biofilm growth noticed after 48 h, Fig. 1A, instead of an increase, could be explained by a detachment of some parts of the biofilm biomass to the surrounding environment, which is a characteristic of mature biofilms.[1,17,21]

Even though the growth conditions used in this study allowed the agglomeration of *S. mutans* on titanium surface at high density, it was not detected a localized corrosion of titanium in the presence of *S. mutans* over a period of 48 h of growth. Nevertheless, the decrease of OCP noticed during electrochemical tests indicated an increase of the chemical reactivity of titanium or else a higher corrosion susceptibility of titanium in the presence of biofilms.

Impedance tests confirmed the OCP results indicating a decrease of the corrosion resistance in presence of *S. mutans* (Fig. 4). The amount of electric charge stored on the titanium surface (in an electric field) immersed in an electrolyte is represented by C_f.[10] The dielectric properties of the passive film can be estimated from the equivalent electrical circuit once an increase of capacitance results in a decrease of the dielectric properties of the passive film. On the other side, Rp_f indicates the ability of the passive film to resist of a current flow on its surface, or else the corrosion resistance of the passive film.[10] The decreased corrosion resistance can be due to the release of lactic acid from *S. mutans* metabolism at high sucrose concentrations to the surrounding environment[1] as shown by pH measurements (Fig. 1A). Also, formic and acetic acids can be released from *S. mutans* metabolism at low sucrose concentration during prolonged periods without nutrients[1] what can contribute to a decrease of pH in the surrounding. The presence of acidic substances, produced by *S. mutans*, on titanium could significantly decrease the pH of the growth medium (Fig. 1A). Thus, the continuous decrease of pH might corrode titanium surfaces located below and around the biofilms. Also, a higher decrease of the corrosion resistance of titanium can be noticed in the presence of mixed biofilms than in the presence of mono-species biofilms.

Considering that the pH of the growth medium was at 4 in presence of high density biofilms, one may assume that the pH within the biofilm could be much lower than the one resulting from a gradual diffusion of acidic substances through the biofilm biomass up to titanium surface.

As reported in previous studies,[9–12] the dissolution rate of the titanium oxide film at low pH is associated to the H^+ concentration in the solution. That results in the formation of hydrated Ti oxides as $Ti(OH)_3^+$, and further in a release of Ti-ions and TiO_2 ultra-fine particles to the surrounding environment. Titanium ions might prevent or decrease bacterial growth due to their toxicity on bacterial cells. In fact, a high concentration of Ti particles at 500 ppm can decrease the microbial cell viability.[1] However, Ti ions and TiO_2 ultra-fine

particles (diameter up to 100 nm) have been reported as toxic for human cells.[32,33] In addition, the release of Ti ions and particles results in a material loss that can promotes failures in titanium-based structures of dental prostheses and implant connections.

5. Conclusions

Concerning the presence of biofilms, the growth of *S. mutans* onto titanium surfaces stabilizes after 2 days of incubation in an enriched medium with a high sucrose concentration. Titanium surfaces covered with a biofilm grown for 2 days, exhibited a capacitive behaviour revealing the presence of a compact titanium passive film without the occurrence of localized corrosion when immersed in artificial saliva. However, the presence of *S. mutans* colonies on the titanium surface negatively affected the corrosion resistance as revealed by the polarization resistance of the titanium passive film. In fact, the decrease of pH caused by acidic substances released from *S. mutans* metabolism can induce the corrosion of titanium-based frameworks and implant-abutment joints during a prolonged period at high sucrose concentration, or in association with other acidic substances and fluorides in the oral cavity.

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