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MONITORING OF MICROBIAL ADHESION AND BIOFILM GROWTH USING ELECTROCHEMICAL IMPEDANCEMETRY

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Abstract

The possibility to use electrochemical impedance spectroscopy to monitor the cell attachment and the biofilm proliferation was tested. In order to identify characteristic events induced on the metal surface Gram-negative (Pseudomonas aeruginosa PAO1) and Gram-positive (Bacillus subtillis) bacteria strains have been studied. Electrochemical impedance spectra of AISI 304 electrodes during cell attachment and initial biofilm growth for both strains were obtained. It can be observed that the resistance increases gradually with the culture time and decreases with the biofilm detachment. So, the applicability of electric cell-substrate impedance sensing (ECIS) for studying the attachment and spreading of cells on a metal surface has been demonstrated. The biofilm formation was also characterized by the use of SEM and confocal laser scanning microscopy and COMSTAT image analysis. The electrochemical results roughly agree with the microscope image observations. The obtained results have been compared to existing data and the possibility to use the method for biofilm characterization was discussed. The ECIS technique used in this study was used for continuous real-time monitoring of the initial bacterial adhesion and the biofilm growth. It provides a simple and non expensive electrochemical method for in vitro assessment of the presence of biofilms on metal surfaces.

Key words: *electrochemical impedance, microbial adhesion, biofilm thickness.*

1. INTRODUCTION

Biofilms are a well recognized phenomenon in process industry. Biofouling and biodeterioration due to biofilm formation are becoming increasingly important since they touch various domains of applications, such as biomedical, industrial marine and environmental. This process is referred to as biocorrosion or microbial influenced corrosion (MIC). Initial bacterial adhesion can be described by the van Oss theory [1,2] in which adhesion is predicted as an interplay of Lifshitz-van der Waals, acid-base and electrostatic interactions between bacteria and substratum. The potential of the interacting surfaces is usually assumed to be constant. However, both surface potential and surface charge density may change during adhesion as it was determined by Poortinga et al. [3].

Charge transfer between bacteria and conducting or semi-conducting materials plays an important role in initial bacterial adhesion. Bacterial cell surface consists of a variety of macromolecules, which contain electrochemical active groups that facilitate charge transfer and exchange [4]. As a result the bacterial surface contains free electrons, which might be exchanged during initial adhesion. The

charge transfer being a fundamental electrochemical process present in chemical and biological systems, electrochemical impedance spectroscopy (EIS) can be successfully used to monitor initial bacterial adhesion and biofilm growth on conducting and semi-conducting materials [4-7] and for the development of sensor devices for continuous real-time monitoring of initial biofilm formation and biofilm growth [8].

The aim of this study was to apply the EIS to monitor the cell attachment and the biofilm proliferation in order to identify characteristic events induced on the metal surface by a Gram-positive Bacillus Subtilis CIP 5265 bacteria strain and a Gram-negative one Pseudomonas auriginosa PAO1. The results are compared to data obtained by scanning electron microscopy (SEM), and confocal scanning laser microscopy (CSLM) to image the cell distribution and obtain quantitative information about the biofilm thickness and its morphology. The possibility to use the charge transfer process involved in the bacterial adhesion for real-time monitoring of the biofilm formation is discussed.

2. THEORITICAL

Application of an electrical perturbation to an electrical circuit induces the appearance of a response. In the EIS, the system response to the application of a sinusoidal signal is studied. This signal is $E = E_o Sin(\omega t)$, where E_o is the signal amplitude, $\omega=2\pi f$ is the angular frequency and f is the signal frequency. The ratio of the Laplace transforms of potential and current, E(s)/i(s), where the parameter $s=j\omega$ called frequency replaces the function of time, is expressed in the units of resistance (Ω), and is called impedance Z(s). In order to simplify the calculations of impedances, the system impedance $Z(j\omega)$, may be represented as

$$Z(j\omega) = R - j\frac{1}{\omega C} \quad (1)$$

with real Z' = R and imaginary Z" = - $1/\omega C$ parts, respectively. Further, the modulus of $Z(j\omega)$ equals:

$$/Z/ = \sqrt{(Z')^2 + (Z'')^2} \quad (2)$$

The result may be presented graphically using two types of plots: complex plane (also known as Nyquist plots) and Bode plots. Complex plane plots are the most often used in the electrochemical literature, while Bode plots are mainly used in the circuit analysis.

Total electrode impedance consists of the contribution of the electrolyte, the electrode-solution interface and the electrochemical reaction taking place on the electrode. For the reaction:

$$Ox + ne^- \underset{k_i}{\overset{k_d}{\leftrightarrow}} Red$$

where k_d and k_i are the potential dependent rate constants for the forward and backward reactions the current is described as

$$i = nF\left[k_d C_O(0) - k_i C_R(0)\right] \quad (3)$$

and f = F/RT. In general, the current is a function of the potential and concentrations and it may be represented as an infinite Taylor series where only first-order derivatives versus concentrations are different from zero. In order to find concentrations, Fick's diffusion equation must be solved for ΔC. For an infinite diffusion layer the impedance is given by:

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$$Z(\omega) = R_t\left(1 + \frac{\lambda}{\sqrt{j\omega}}\right) \quad \text{with} \quad \lambda = \frac{k_d}{\sqrt{D_{Ox}}} + \frac{k_i}{\sqrt{D_{Red}}} \tag{4}$$

In this manner the total electrode impedance consists of the solution resistance, R_e, in series with the parallel connection of the double-layer capacitance, C_d, and faradaic impedance replaced by the charge-transfer resistance R_t and the Warburg impedance W. This is the so called Randles model. The model for a cell where polarization is due to a combination of kinetic and diffusion processes is shown in Fig.1.

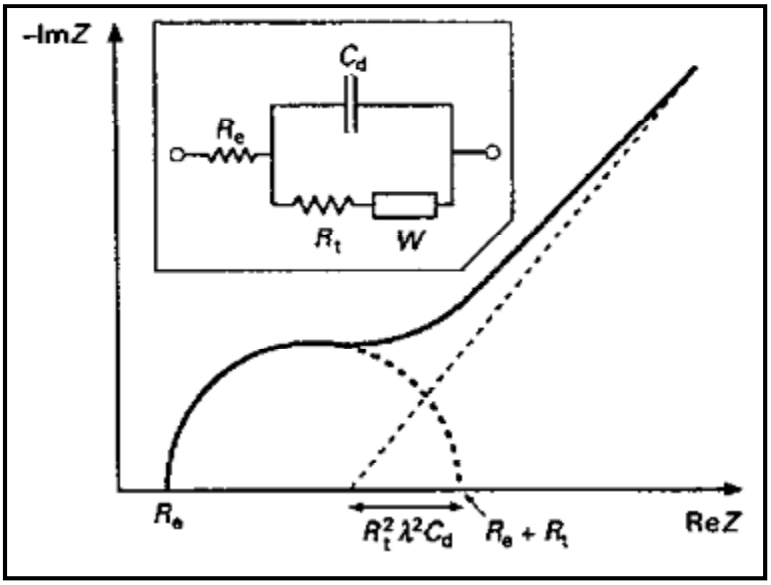

Fig. 1 Complex plane plot according to the Randles model

3. MATERIALS AND METHODS

3.1. Support used

To study the microbial adhesion AISI 304 stainless steel grids and coupons (thickness 1.1 mm) were used. The support hydrophobicity was determined by contact angle measurement after ultrasonically cleaning in acetone, ethanol and deionised water. Contact angles were obtained using the sessile drop method with a Digidrop Model MSE contact angle analyser. This instrument consists of a CCD video camera, multiple dosing/micro-syringe units and a temperature controlled environmental chamber. The drop image was processed by an image analysis system (Windrop), which calculates both the left and the right contact angles from the shape of the drop with an accuracy of ± 0.1°. Three test liquids were used as a probe for surface free energy calculations: distilled water, diiodomethane and formamide.

The supports exhibit a hydrophobic character (water contact angle of 80.7°) and have a surface free energy of 41.1 $mJ.m^{-2}$ (γ^{LW} = 40.5 $mJ.m^{-2}$, γ^{AB} = 0.6 $mJ.m^{-2}$). These parameters have been used to select the microorganisms.

3.2. Bacteria used

In this study, a Gram-positive bacteria strain Bacillus Subtilis CIP 5265 (with a thick layer of peptidoglycan in the cell wall) and a Gram-negative bacteria strain Pseudomonas aeruginosa PAO1gfp containing the plasmid pSMC21 were used. The plasmid constitutively expresses gfp, which label the strain for microscopy studies. This P.aeruginosa strain does not produce an important amount of exopolysaccharides (EPS). The organisms were defrosted and cultured in Luria-Bertani (LB) medium, containing Tryptone - 10 $g.l^{-1}$, Yeast extract - 5 $g.l^{-1}$and NaCl - 10 $g.l^{-1}$ at pH=7 and 37°C in a rotary shaker (180 rpm). For P. aeruginosa PAO1gfp, medium culture was supplemented with ampicillin and kanamycin. The various samples were exposed to the cell suspension in flasks in a rotary shaker at 37°C. The culture medium was changed every 24h, and cultivation of the biofilm was continued in the same flasks. At different time intervals three samples were taken out and treated with sterile deionised water to remove the non-adhered bacteria.

3.3. Monitoring of cell attachment and biofilm growth

The experiments were conducted at constant temperature (37°C). The dynamics of microbial colonisation was monitored using scanning electron microscopy (SEM), confocal scanning laser microscopy (CSLM) and electrochemical impedancemetry. Microscopy provides information about the morphology of microbial cells and colonies, their distribution on the surface, the presence of EPS, etc. CSLM pictures were taken at different times of cultivation and were used to provide three-dimensional information on the biofilm/metal surface interactions. Using these pictures, the biofilm structures were quantified using a COMSTAT image analysis [9,10], which comprises ten features for quantifying three-dimensional biofilm image stacks.

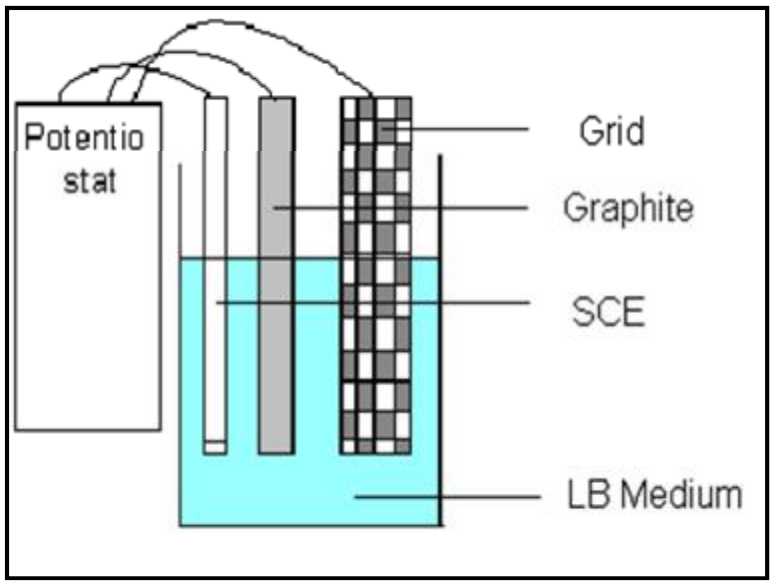

Fig.2. Schematic diagram of the electrochemical cell

Electrochemical impedance experiments have been performed using an electrochemical workstation with a one-compartment three-electrode cell. A saturated calomel electrode (SCE) and a graphite electrode served as reference and counter electrode, respectively. The grids (colonized and non-

colonized) were used as working electrodes connected to a potentiostat (Voltalab 40- Radiometer Analytical Instruments, France). A homemade single-compartment cell shown in Fig.2 was used in these experiments. A constant working electrode geometrical area has been ensured for different electrodes in order to improve the measurement reproducibility and the reliability of the experimental results.

4. RESULTS

4.1. Hydrophobicity

Microbial surface hydrophilicity or hydrophobicity can be evaluated using ΔG_{131}^{TOT}, being the total free energy of interactions between the microbial cells (1) immersed in water (3), determined as the sum of ΔG_{131}^{LW}, i.e. Lifshitz-Van der Waals interaction free energy and ΔG_{131}^{AB}, i.e. Lewis acid-base interaction free energy [8]:

$$\Delta G_{131}^{TOT} = \Delta G_{131}^{LW} + \Delta G_{131}^{AB} \quad (5)$$

$$\Delta G_{131}^{LW} = -2\left(\sqrt{\gamma_3^{LW}} - \sqrt{\gamma_1^{LW}}\right)^2 \quad (6)$$

$$\Delta G_{131}^{AB} = -4\left(\sqrt{\gamma_1^{+}} - \sqrt{\gamma_3^{+}}\right)\left(\sqrt{\gamma_1^{-}} - \sqrt{\gamma_3^{-}}\right) \quad (7)$$

Bacterial hydrophobicity was determined by water contact angle measurement on bacterial lawns. According to Table 1 bacteria exhibit a predominant hydrophilic surface with contact angles of 40 degrees. Our results concerning the contact angles appear to be very close to those obtained by Logan [11]. The bacteria exhibit a monopolar surface, and the component of the surface free energy γ^- plays the most important role among other surface thermodynamic parameters. The surface free energy of the bacteria is 45 $mJ.m^{-2}$ so only substrates exhibiting lower surface free energy can be significantly colonized. That confirms the choice of the AISI 304 stainless steel grids as suitable support for this study.

Bacterial adhesion to solid surfaces can be evaluated using ΔG_{132}^{TOT}, the total free energy of interactions between bacterial cells (1) and the substrate (2) immersed in water (3), is determined as the sum of Lifshitz-Van der Waals interaction energy ΔG_{132}^{LW}, Lewis acid -base interactions free energy ΔG_{132}^{AB}, and electrostatic interaction free energy ΔG_{132}^{EL}:

$$\Delta G_{132}^{TOT} = \Delta G_{132}^{LW} + \Delta G_{132}^{AB} + \Delta G_{132}^{EL} \quad (8)$$

$$\Delta G_{132}^{LW} = -2\left(\sqrt{\gamma_3^{LW}} - \sqrt{\gamma_1^{LW}}\right)\left(\sqrt{\gamma_2^{LW}} - \sqrt{\gamma_3^{LW}}\right) \quad (9)$$

$$\Delta G_{132}^{AB} = 2\left(\sqrt{\gamma_3^{+}}\left(\sqrt{\gamma_1^{-}} + \sqrt{\gamma_2^{-}} - \sqrt{\gamma_3^{-}}\right) + \sqrt{\gamma_3^{-}}\left(\sqrt{\gamma_1^{+}} + \sqrt{\gamma_2^{+}} - \sqrt{\gamma_3^{+}}\right) - \sqrt{\gamma_1^{+}\gamma_2^{-}} - \sqrt{\gamma_1^{-}\gamma_2^{+}}\right) \quad (10)$$

This is the case of the support studied. The total free energy of interactions ΔG_{132}^{TOT} has been evaluated. The obtained value (given in Table 1) is lower than the value of ΔG_{131}^{TOT} ($\Delta G_{132}^{TOT} < \Delta G_{131}^{TOT}$) so bacterial adhesion to the substratum can be observed.

The charge transfer is an omnipresent process in biological systems. As it is involved in bacterial cell adhesion, electrochemical impedance spectroscopy can be used in the investigation of the biofilm formation and growth on conducting surfaces. Fig. 3 shows typical electrochemical impedance spectra for the PAO1 and BS bacteria strains presented as Nyquist plots (Z_{im} vs. Z_{re}) of an AISI 304 stainless steel grid before and after colonization recorded in a LB culture medium. In the control experiment,

the stainless steel grid was incubated in culture medium in absence of cells, and then electrochemical impedance measurements were performed by using these samples as working electrodes. It was possible, in this manner, to take into account the non-specific adsorption of substances from the culture medium onto the metal surface.

Table 1. Contact angles and surface free energy components ($mJ.m^{-2}$) of the microbial strains

	Contact angle	γ^{AB}	γ^{LW}	γ^{+}	γ^{-}	E_T	ΔG_{131}^{TOT}	ΔG_{132}^{TOT}
B.subtilis	34	18,32	30,2	1,8	46.6	48,3	24,99	8,1
P.aeruginosa	40	4,45	32,70	0,1	49.6	45,4	35,53	11,28

4.2. EIS and microscope monitoring of cell attachment and biofilm growth on AISI 304 electrodes

As shown in Fig.3 the adhesion process is presented by a typical Randles model for a cell where polarization is due to a combination of kinetic and diffusion processes. The electron-transfer resistance increases significantly after 7 days of bacterial colonization. The significant increase observed is related to existence of cells on the AISI 304 stainless steel surface. At the same time no significant difference can be detected concerning the initial bacterial adhesion of both strains. It can be concluded, that the electrochemical method detects the initial bacterial adhesion but cannot differentiate various bacterial strains.

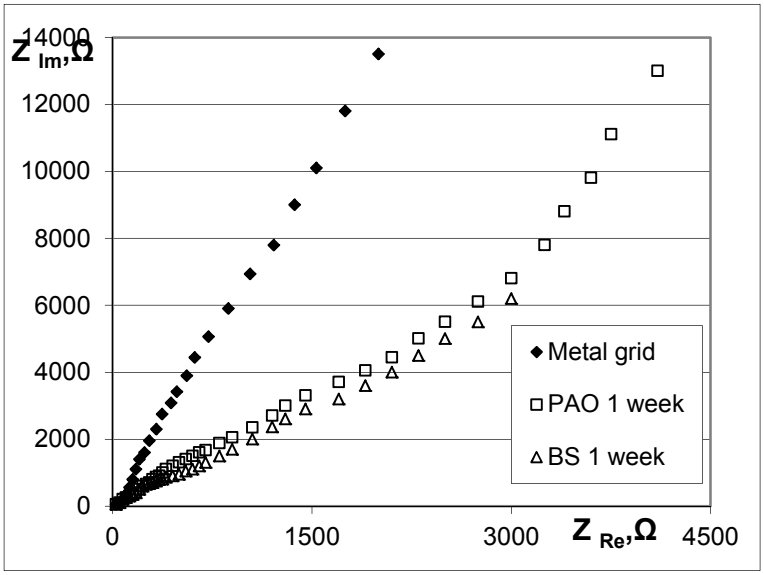

Fig.3 Typical Nyquist plots for the electrochemical impedance measurements for a bare support (◊) and colonized with P.aeruginosa and B.subtillis electrodes

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At the same time, bacterial adhesion on metal grid samples was also observed using a Leica TCS SP II confocal scanning laser microscope (Leica Lasertechnik, Heidelberg, Germany). The CSLM pictures of *Ps.aeruginosa* and *Bacillus subtillis* after 1 week biofilm growth given in Fig. 4 show an important difference in the biofilm structure.

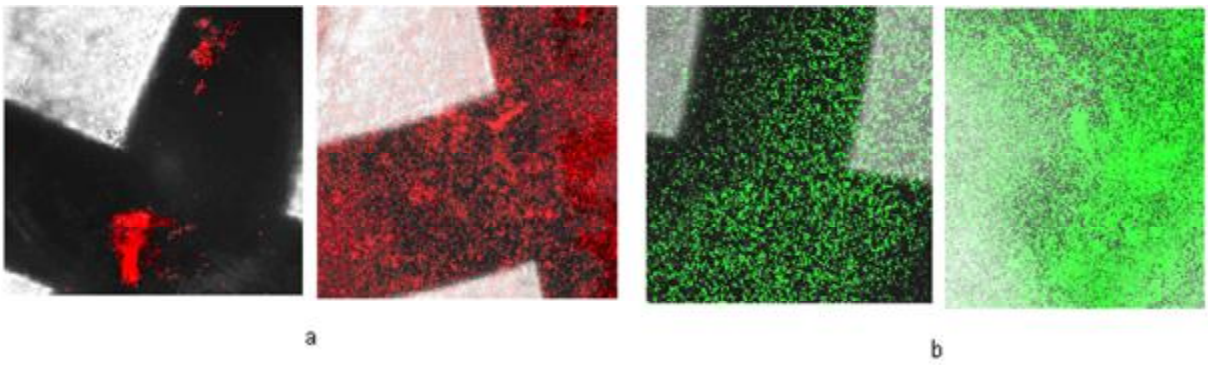

Fig. 4 CSLM pictures of *B.subtillis* (a) and *Ps.aeruginosa* (b) biofilms after 24 hours and 1 week biofilm growth

To confirm this difference SEM pictures have been taken during the cultivation course. Fig 5 shows the biofilm growth observed using SEM pictures. From the beginning, the *P.aeruginosa* adhesion takes place on some points of the surface of the support. The biofilm depth is not uniform. After 72h of cultivation course, a thin biofilm monolayer is formed and in the following days, the cells grow unceasingly and a multilayer film is observed. The *B.subtillis* bacterial strain forms colonies and these colonies spread on the surface without forming a uniform layer.

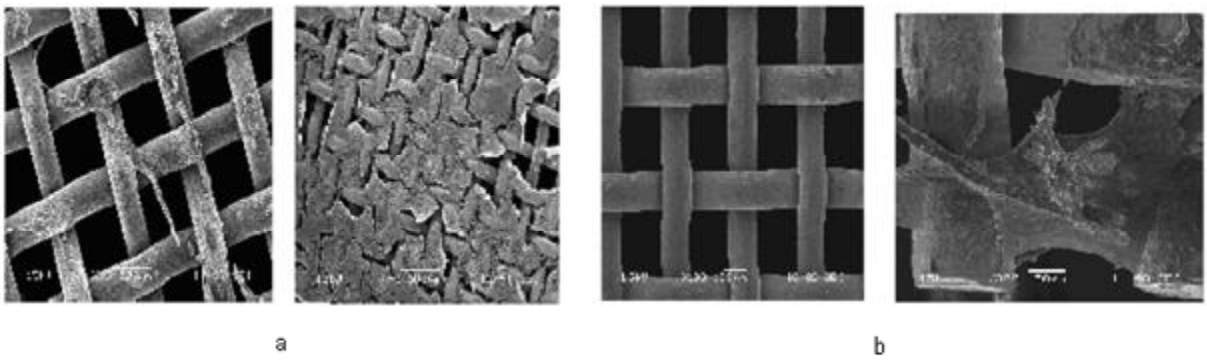

Fig. 5 SEM pictures of P.aeruginosa a) and B.subtillis b) biofilm after: 9h and 72h of cultivation

The obtained results show, however, that charge transfer is involved in bacterial cell adhesion and that electrochemical impedance spectroscopy can be used in the investigation of the biofilm formation and growth on conducting surfaces. Fig. 6 illustrates the variations of electrochemical impedance spectra for the PAO1 bacteria strain presented as Nyquist plots of an AISI 304 stainless steel grid before and after colonization recorded in a LB culture medium. It can be observed that bacterial adhesion on the

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electrode surface affects the impedance of the system. When initial bacterial adhesion occurs the impedance magnitude is found to decay exponentially as the biofilm is formed.

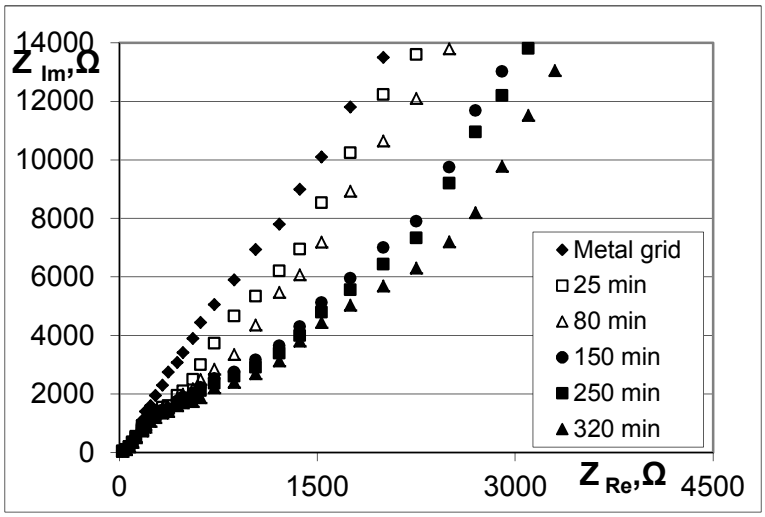

Fig. 6 Nyquist plots for the electrochemical impedance measurements for a bare support and colonized with P.aeruginosa electrodes (different colonization times)

If an image analysis is performed, an important difference between both bioadhesion mechanisms can be detected. Fig. 7 illustrates the variations of the impedance magnitude as a function of time during the cell attachment and the initial biofilm growth for the *Pseudomonas aeruginosa* strain.

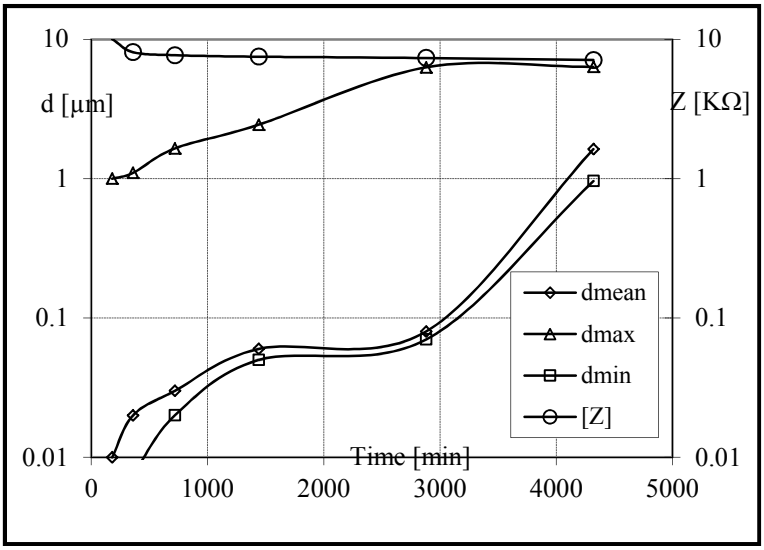

Fig. 7 Variations of biofilm depth and impedance magnitude as function of time

(d_{mean} – mean biofilm depth; d_{max} – maximal biofilm depth; d_{min} – minimal biofilm depth)

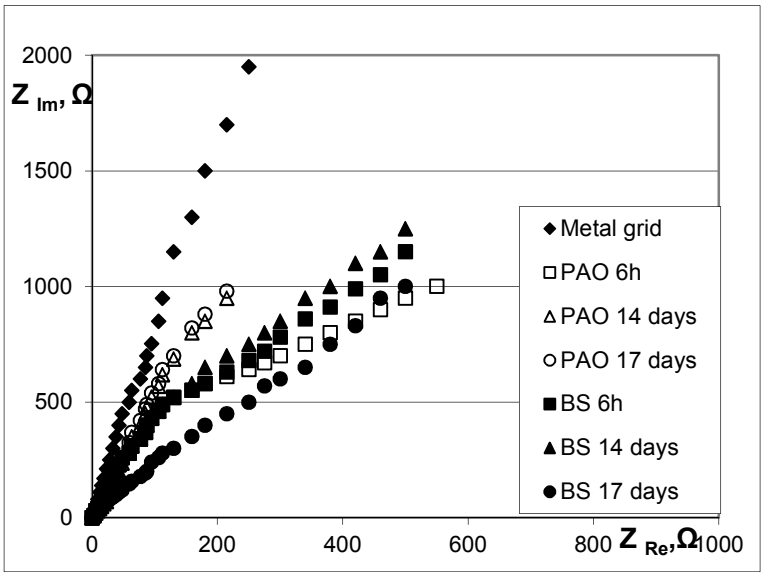

Fig. 8 Nyquist plots for the electrochemical impedance measurements for a bare support and colonized by *P.aeruginosa* and *B.subtillis* electrodes (aged biofilms)

It can be observed, that bacterial adhesion on the electrode surface affects the impedance of the system. The difference between the maximal and the mean biofilm depth shows that the initial colonization is not uniform and takes place in function of the surface roughness. Further, the minimum biofilm depth increases and equals the mean biofilm depth. The biofilm becomes more uniform and the impedance magnitude decreases only slightly. If the same analysis is performed on the *Bacillus subtillis* the maximal biofilm depths is very important (106 µm), while the minimum equals 0.001 µm and remains low and constant. Even aged biofilm does not exhibit higher minimum depths. In fact, aged *Bacillus subtillis* biofilms remain stable and show little evolution with time, while the *Pseudomonas aeruginosa* biofilm is easy to erode when aged and thick, as it can be seen in Fig. 8.

5. DISCUSSION

Some authors [7, 12] have shown that the adsorption of bacteria on the electrode surface can affect dramatically the variations of the real and imaginary impedance components at a detection time. In order to investigate the possibility to differentiate various bacterial strains using the imaginary and the real impedance, these parameters are plotted against the deposition time as shown in Fig. 9a and 9b. The data are obtained at a fixed low frequency equal to 147.1 mHz. This frequency allows the comparison between our data and those obtained by Bayoudh et al. [12]. To take into account the initial conductivity of the culture medium, the results are presented in the dimensionless form Z/Zo .

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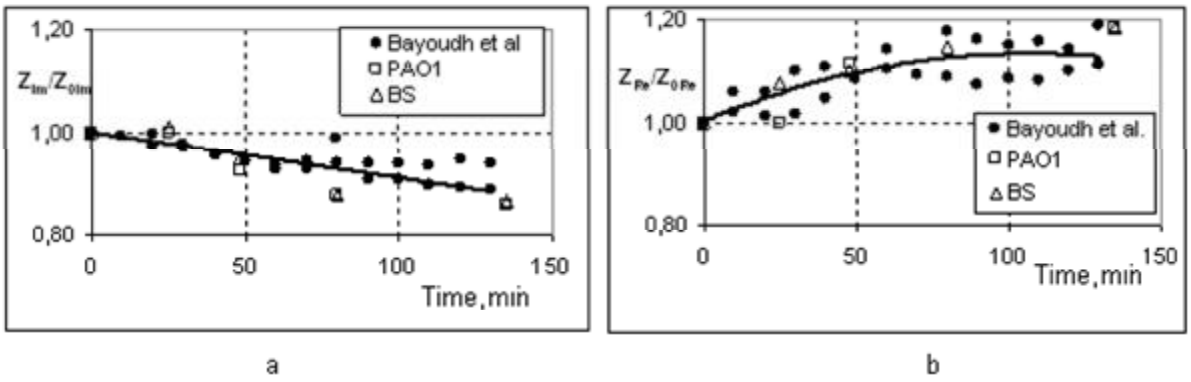

Fig.9 Variations of the real (a) and imaginary (b) impedance as function of time

As it can be seen, the decrease in the impedance magnitude (Fig.6) is determined predominantly by the decrease in its imaginary component, since the real impedance component increased only slightly. The imaginary impedance originates from the double charged layer capacitance, reflecting well the adsorption-adhesion process on the substratum surface [6]. This indicates that this parameter plays an important role in bacterial adhesion. At the same time, the comparison with other data shows that in all cases the decrease in the imaginary impedance and the lower increase in the real impedance can be fitted as a linear decrease resp. polynomial increase of these parameters. The given relationships are independent on the bacterial strain. It is important to note that Gram-positive and Gram negative strains show the same comportment and so it can be concluded that the electrochemical impedance spectroscopy can successfully detect the initial bacterial adhesion and the biofilm growth, but cannot give information about the bacterial strain and the biofilm character and thickness.

A typical electrochemical impedance spectrum presented in the form of a Nyquist plot includes a semicircle region lying on the Z_{Re} axis observed at higher frequencies corresponding to the electron-transfer-limited process, followed by a linear part at lower frequencies representing the diffusion-limited electron-transfer process. Usually, the electron-transfer kinetics and diffusional characteristics can be extracted from the spectra. The attachment of the cells and the development of the biofilm on the metal surface are expected to retard the interfacial electron-transfer kinetics and increase the electron-transfer resistance. As shown in Fig. 3 the charge-transfer resistance increases significantly after 7 days of bacterial colonization. The significant increase observed is related to existence of cells on the AISI 304 stainless steel surface. As, the method is based on a resistance measurements, it is evident that abiotic processes in the culture medium can influence the obtained results. The control experiment takes into account for these phenomena.

Biological cells often act as insulating particles when attach and grow on substrates, and therefore the existence of cells on the electrode surface would undoubtedly inhibit the electron transfer between the redox probe in the solution and the working electrode. This result indicates that EIS is capable of monitoring the change in charge-transfer resistance resulting from the growth of bacteria on the electrode surface. The *Pseudomonas aeruginosa* strain which is an EPS producer colonizes the entire substratum very rapidly (substratum coverage of 80-95% on all days) and therefore showed good biofilm formation despite of its relative hydrophobicity. Obviously the bacteria outer surface and polymer production have some influence on the adhesion process as it was mentioned by Liu et al [13]. Our results can also be compared to results obtained by Klausen [10] and Heydorn [9] concerning the biofilm structure, his thickness and surface to volume ratio of *Pseudomonas*

aeruginosa grown on various media. In all cases the substratum coverage is very high but the biofilm structure depends on the nutritional and environmental conditions as mentioned by Klausen et al [10].

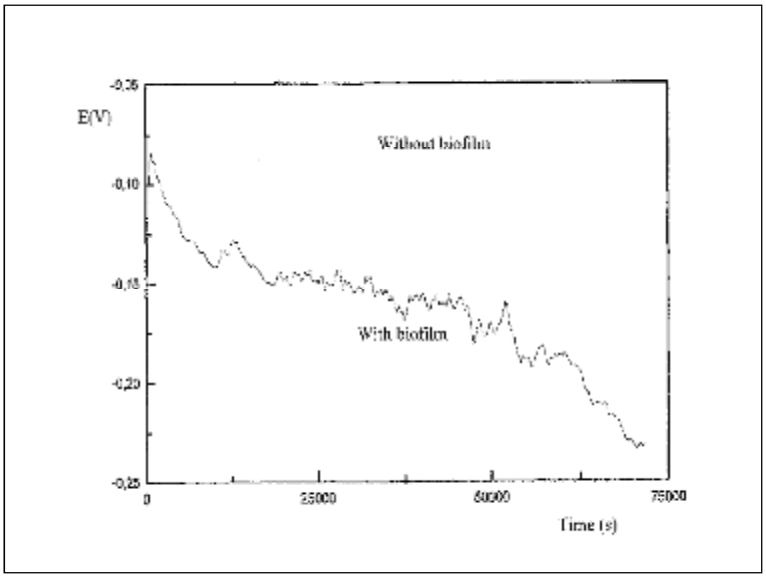

Fig. 10 Real-time monitoring of the initial biofilm formation and the biofilm growth of *Ps.aeruginosa* biofilm (comparison between support with and without biofilm)

This is not the case of the *Bacillus subtillis* strain. It is most hydrophilic and has lower total free energy of interactions ΔG_{132}^{TOT} so cell adhesion must be more rapid. At the same time, the BS strain does not produce EPS. Taking all this into account, we can consider that during the early cultivation periods, cells are attached on the metal surface resulting in an electron-transfer barrier between the redox probe and the electrode surface. They form colonies that grow during cultivation. Initially, the free metal surface is relatively important so this barrier is not very important. With the increase in the number of attached cells, the charge-transfer barrier increases. The difference between both strains appears after about 24 hours of cultivation. In the case of *Pseudomonas aeruginosa*, the number of attached cells is important and their metabolic activities contribute to a uniform biofilm formation, while the *Bacillus subtillis* strain increases the number of colonies without changing the biofilm structure. These activities do not influence significantly the electron-transfer barrier between the electrodes. The *Pseudomonas aeruginosa* mature biofilm once formed, it deepens more and more which results in a larger and larger electron-transfer barrier between the electrodes. Obviously, the cells have been allowed to grow on the support for enough time to reach density inhibition and to form considerable amount of EPS. After 1-2 weeks of cultivation the curves obtained with both strains are not identical. The BS strain remains firmly attached on the substratum, while part of the *Pseudomonas aeruginosa* cells are reversibly attached, do not come in direct contact with the substratum surface and are held only by temporary cellular bonds. These bonds can be broken by changes in flow or cell motility. This fact can justify the results given in Fig.8.

Obviously, the electric cell-substrate impedance sensing (ECIS) can be a powerful tool for characterizing initial bacterial adhesion, the presence of mature biofilm and to quantify the effect of biocide injection or sterilization on existing biofilm. The applicability of ECIS detecting the

attachment and spreading of cells on a metal surface can be demonstrated for the case of *Pseudomonas aeruginosa* strain. Quantitative information about cell growth has been obtained with this method and shown in Fig. 10 where potentials of zero charge for AISI 304 stainless steel surfaces without and with biofilm are compared. The results show that the ECIS technique used in this study can be used for continuous real-time monitoring of the initial biofilm formation and the biofilm growth. It provides a simple and non expensive electrochemical method for in vitro assessment of the presence of biofilms on metal surfaces.

6. CONCLUSION

The applicability of electric cell-substrate impedance sensing (ECIS) for studying the attachment and proliferation of cells on a metal surface has been demonstrated for the *Pseudomonas aeruginosa* and *Bacillus subtillis* strains. Quantitative information about cell growth has been obtained using this method. The results are compared to SEM and CSLM pictures. The electrochemical results roughly agree with the microscope image observations. The ECIS technique can be used for continuous real-time monitoring of initial biofilm formation and biofilm growth. It provides a simple and inexpensive electrochemical technique for in vitro assessment of the presence of biofilms on metal surfaces.

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