Biofouling, August 2003 Vol 19 (4), pp 215–222

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The Use of Cyclic Voltammetry to Detect Biofilms formed by *Pseudomonas fluorescens* on Platinum Electrodes

MARIA JOÃO VIEIRA[a,*], ISABEL ALEXANDRA PINHO[a], SALOMÉ GIÃO[a] and MARIA IRENE MONTENEGRO[b]

[a]*Centro de Engenharia Biológica-IBQF, Universidade do Minho, 4710-057 Braga, Portugal;* [b]*Centro de Química-IBQF, Universidade do Minho, 4710-057 Braga, Portugal*

(Received 11 November 2002; in final form 15 January 2003)

The development of an electrochemical detector to monitor the *in situ* formation of biofilms is described. The detector consisted of an electrochemical cell containing three electrodes, whose response to the application of a potential profile to the working electrode was sensitive to the amount of biofilm present on the surface. The electrochemical technique used was repetitive cyclic voltammetry. Differences between the response of the uncolonised electrode and after *Pseudomonas fluorescens* biofilms of different ages were grown on its surface were determined. The results show that cyclic voltammetry applied to platinum electrodes can be used to detect young biofilms. The development of the shape of the voltammogram as the potential is cycled may constitute a means of providing information on the coverage of the surface. Observation of the platinum electrodes before and after the electrochemical measurements showed that even after 30 min of recycling, most of the cells were still adhered to the surface, although some may have lost viability.

Keywords: biofilms; *Pseudomonas fluorescens;* monitoring; biofouling; electrochemistry; cyclic voltammetry

INTRODUCTION

Biofouling, the unwanted accumulation of biofilms on industrial equipment occurs due to favourable nutrient and temperature conditions and the availability of microorganisms. Biofouling is a costly problem, and optimization of the control of unwanted biofilm accumulation is of primary importance. Once established, the strategies used to eradicate biofilms rely on the application of chemical agents and on cleaning procedures, which always have associated high costs (Flemming, 1997). The early detection of biofouling may allow the application of more effective strategies to eradicate biofilms. The need for on-line monitoring techniques that are reliable, easy to implement and inexpensive is widely recognised (Licina *et al*., 1994; Flemming *et al*., 1998; Klahre & Flemming, 2000). The various on-line monitoring methods described in the literature have inherently different sensitivities and can give different types of information about the biofilm. Recently, Flemming (2002) proposed a classification of monitors on three levels according to the type of information they are able to give. Some monitors give reliable results on the kinetics of biofilm formation and variation in the thickness and mass of the deposit, *e.g*. the fibre optic device, the differential turbidity measurement device (Flemming *et al*., 1998), the heat transfer resistance device (Melo & Vieira, 1999), the infra-red monitor (Tinham & Bott, 2002), the electrochemical method probe 'Biox' (Mollica & Cristiani, 2002). A second type of monitor distinguishes between biotic and abiotic components of a given deposit, such as FTIR-spectroscopy specific for amino bands (Flemming, 2002). Finally, the third level may give information on the chemical nature of the biofilm and metabolic activities of the microorganisms *e.g*. the FTIR-ATR spectroscopy flow cell (Flemming *et al*., 1998).

Electrochemical methods provide a convenient means of detecting the early stages of biofilm formation in heat exchangers and water systems, as demonstrated by the large number of investigations on surface fouling by bacteria based on electrochemical techniques (Mittelman *et al*., 1992; Licina *et al*., 1994; Cristiani *et al*., 1996; Illsley *et al*., 1997; Cachet *et al*., 2000). In these studies, one or more electrochemical parameters such as the current density, potential difference, impedance, potential

*Corresponding author; fax: 00 351 253 678986; e-mail: mjv@deb.uminho.pt

ISSN 0892-7014 print/ISSN 1029-2454 online
DOI: 10.1080/0892701031000010080

noise, potential resistance (Cristiani *et al*., 1996) or the charge density of the surface before and after biofilm formation (Illsley *et al*., 1997) is measured and, in some situations, related to the accumulation of biofilm on the surface. The sensor described by Mittelman *et al*. (1992) is a flow cell where the on-line electrochemical monitoring of the surface potential change is recorded as a function of the colonization of the surface by bacteria. Licina *et al*. (1994) developed the BIoGEORGE^{TM} Probe to monitor changes in electrochemical reactions produced by biofilms on stainless steel (SS) electrodes by using polarization of the electrodes. Cristiani *et al*. (1996) reported that the 'Biox' probe can monitor biofilm growth on-line by evaluating changes in the kinetics of the electrochemical processes occurring on the metal surfaces induced by the deposition of microorganisms. This electrochemical sensor is obtained by coupling SS electrodes with sacrificial anodes and the formation of biofilm on the SS electrodes influences the galvanic current measured (Mollica & Cristiani, 2002). Illsey *et al*. (1997) noticed that by using cyclic voltammetry applied to platinum electrodes colonized with bacteria, the surface charge density changed due to the presence of the bacteria on the surface. Cachet *et al*. (2000) used a conductive transparent electrode, where they recorded current-potential curves together with an examination of the surface using a video camera to obtain the surface coverage.

The present study describes the development of an electrochemical detector based on cyclic voltammetry on platinum electrodes, to monitor the formation of biofilms *in situ* in batch systems. The detector consisted of an electrochemical cell containing three electrodes, whose response to the application of a potential profile to the working electrode was highly sensitive to the amount of biofilm deposited on the surface.

When cyclic voltammetry was applied to a platinum electrode immersed in air-free aqueous sulphuric acid, and the electrode surface was clean, the current passing through the cell plotted *vs* the electrode potential, when the potential limits of the scan are 0 and 0.4 V *vs* a standard hydrogen electrode, is the cyclic voltammogram depicted in Figure 1 (Bard & Faulkener, 1980). Peaks for the formation and oxidation of both adsorbed hydrogen and adsorbed oxygen are shown. Peaks in regions 1 and 4 correspond to the oxidation and formation of adsorbed hydrogen, respectively, while peaks 2 and 3 correspond to the adsorption and desorption of oxygen. This is a well-known pattern and details on this voltammogram depend on the scan rate, the reverse potential, the pre-treatment of the electrode surface and on the solution composition.

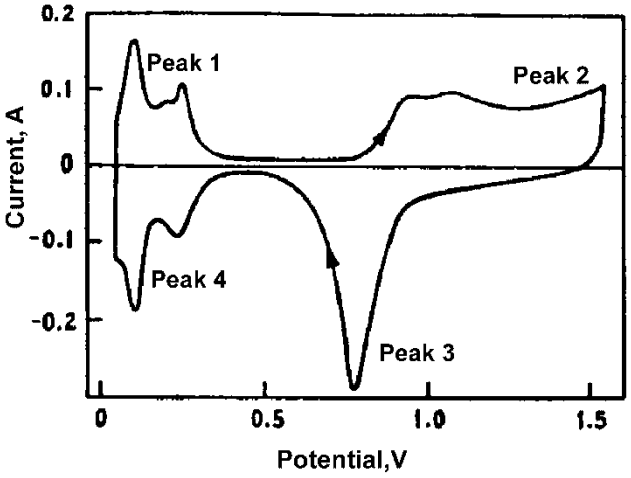


FIGURE 1 Cyclic voltammogram for a platinum electrode in 0.5 M H_2SO_4. Peak 1 = oxidation of adsorbed hydrogen; peak 2 = adsorption of oxygen; peak 3 = desorption of oxygen; peak 4 = adsorbed hydrogen formation.

MATERIALS AND METHODS

Electrochemical Monitoring

The electrochemical technique used was repetitive cyclic voltammetry applied to a platinum electrode with and without biofilm on the surface. A triangular potential sweep was continuously applied to the platinum electrode (working electrode, WE) and the current caused by the breakdown of water molecules was recorded as a function of the applied potential (Pletcher, 1991). The potential profile is, therefore, a linear function of time and can be described as $E_{app} = E_i \pm vt$, where E_{app} is the applied potential at a time t, E_i is the initial potential and v is the scan rate in V s^{-1}. At a pre-set value, the scan rate is reversed and the potential is scanned to the initial value. This cycle can be repeated as many times as required.

The platinum working electrodes (WE) were the substratum for biofilm formation. The WE were 1 mm diameter platinum discs (area of the sensor 7.85×10^{-7} m^2), prepared by sealing a platinum wire into a glass tube and polishing the surface of the cross section using a polishing cloth and alumina powder. The internal end of the platinum wire was sealed to a copper wire that provided the external contact. The reference electrode (RE) was a Metrohm silver/silver chloride electrode (reference 6.0702.100) and all the data reported here are relative to this reference. The auxiliary electrode (AE) was a platinum spiral. After polishing, the electrode was cleaned with deionised water and polished again using a clean polishing cloth.

The batch electrochemical cell consisted of a three-compartment 15 ml all-glass cell, which contained the platinum WE, the platinum AE and the silver/silver chloride RE (Figure 2). For the experiments carried out, a phosphate buffer (pH 7; 0.2 mol l^{-1} Na_2HPO_4

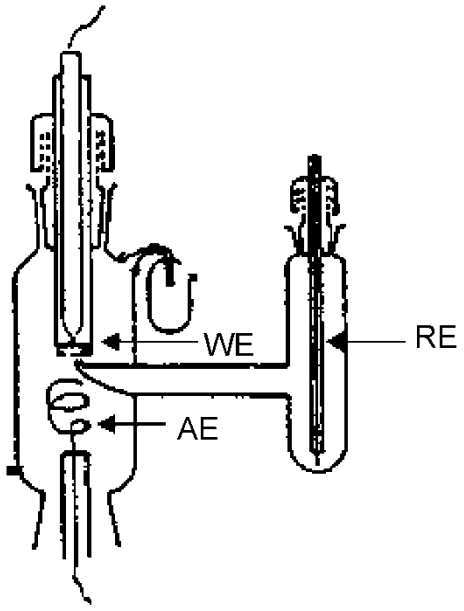


FIGURE 2 Schematic view of the glass electrochemical cell, with a volume of 15 ml. It comprises 3 electrodes, the platinum working electrode (WE) where the biofilm was previously formed, the auxiliary electrode (AE) and the reference electrode (RE).

and 0.2 mol l^{-1} NaH_2PO_4) was chosen as the electrolyte since it provided an appropriate medium for the bacteria and a convenient solution pH.

The electrochemical experiments were carried out using a potentiostat Autolab type PGSTAT 20, Ecochemie. Each electrode was subjected to the electrochemical treatment by immersion in the solution of interest; the potential was recycled between the appropriate limits.

All chemicals used were of analytical grade. Solutions were made up with deionised water (resistivity 18 MΩ cm^{-1}) from a Barnstead E-pure purifier. The solutions were not degassed in order to get similar conditions to those observed in real systems. The voltammograms were recorded using the data acquisition program GPES 4.6.

For each condition tested, the electrochemical measurements were carried out using eight platinum electrodes and a comparison was made between cyclic voltammograms obtained with the uncolonized platinum surfaces and after biofilms of different ages were grown on the electrodes. Prior to their utilization, the electrodes were degreased with ethanol, rinsed twice with sterile deionized water and sterilized using ethanol. Afterwards they were rinsed with sterile deionized water before being cleaned electrochemically, by recycling the potential between -0.5 and 1.0 V for 30 min at 250 mV s^{-1}.

Microorganism and Cell Growth

Pseudomonas fluorescens (ATCC 13525), a Gram-negative aerobic bacterium, was used as a biofilm producer (Pereira & Vieira, 2001). A culture of *P. fluorescens* was grown in batch mode in a 1.0 l glass fermenter, at $27 \pm 1^{\circ}C$, suitably aerated and magnetically agitated, until the culture reached the exponential growth phase. At this point, the fermenter was continuously fed with 0.1 l h^{-1} of a sterile nutrient solution consisting of 5 g glucose l^{-1}, 2.5 g peptone l^{-1} and 1.25 g yeast extract l^{-1}, in phosphate buffer at pH 7 (0.2 mol l^{-1} Na_2HPO_4 and 0.2 mol l^{-1} NaH_2PO_4). This fermenter was used as a continuous source of bacteria.

Biofilm Set-up

A second fermenter (volume 1.0 l) was used to form biofilms on the surface of the platinum electrodes. It was inoculated with the bacterial cells grown in the first fermenter and was operated in batch mode until the exponential phase of growth was reached. The system was continuously fed with sterile medium containing 50 mg l^{-1} glucose, 25 mg l^{-1} peptone and 12.5 mg l^{-1} yeast extract in phosphate buffer (pH = 7, ionic strength of 0.2 mol l^{-1}), well agitated and aerated. The reactor was continuously fed with the bacterial cells grown in the first fermenter. The dilution rate of 0.8 h^{-1} ensured that biofilm growth predominated over that of planktonic cells (Vieira *et al.*, 1993).

The platinum WEs were immersed in the fermenter at different intervals of time (eight electrodes were used for each condition tested) in order to grow biofilms on the surface of the electrodes. These electrodes with biofilms on their surfaces were used to carry out the electrochemical measurements.

Epifluorescence Observations

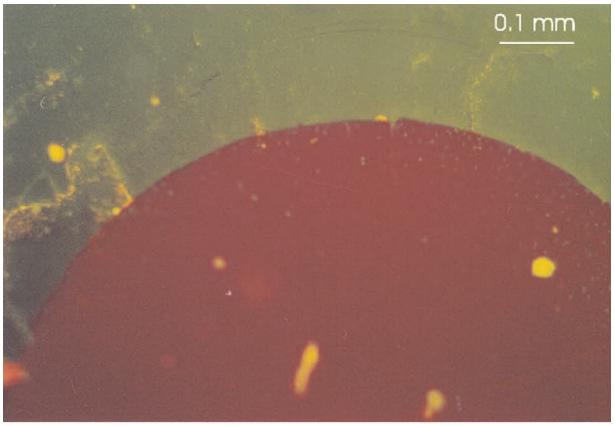
Some platinum electrodes were visualized under epifluorescence microscopy after staining the surface with the *Bac*ligth viability kit (Molecular Probes). The two *Bac*Ligth stains, SYTO 9 and propidium iodide, dissolved in DMSO, were mixed together (130 μl + 130 μl), dropped on the surface and incubated for 15 min. The electrodes were observed under epifluorescence (viable cells fluoresced green, whilst dead cells fluoresced red).

RESULTS AND DISCUSSION

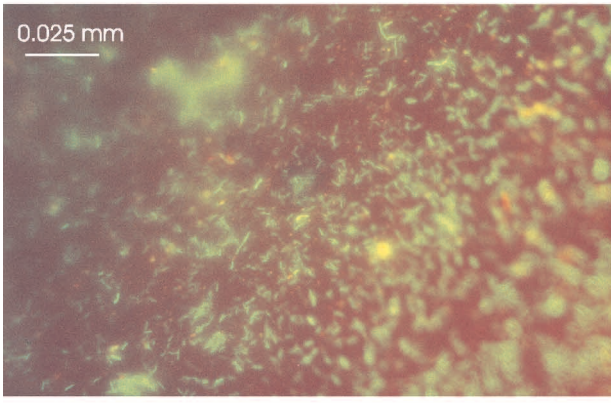
A series of experiments was conducted to determine the extent of biofilm formation on the surface of the platinum electrodes. Figure 3 gives the direct verification of the accumulation of biofilm on the electrodes, after their staining with the Live/Dead viability kit.

Figure 3a shows an uncolonized surface (also stained with the fluorochromes) where the circular

platinum surface is clearly distinguishable from the glass surface that appears brighter. A 2-h biofilm (Figure 3b) still has discrete microorganisms and microcolonies on the surface, which are mainly alive since they appear green. Figure 3c shows a 7-d biofilm, with a very heterogeneous structure. Abundant amounts of polysaccharides can be observed on some areas of the surface, constituting EPS fronds (the fluorochromes used stained not only the bacterial cells but also the EPS matrix), masking the bacterial cells (Keevil, 2002). Other zones still have discrete bacterial cells.



(a)



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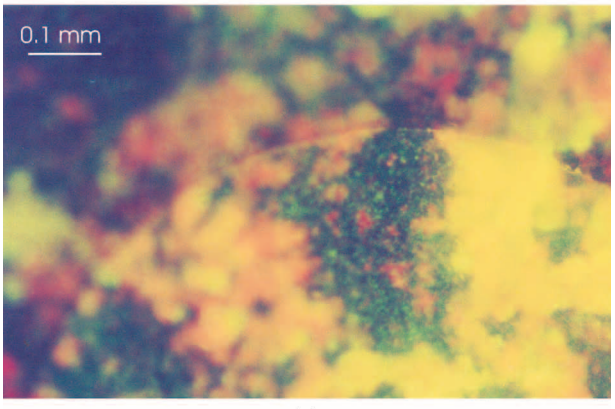

(c)

FIGURE 3 Epifluorescence microphotographs of the platinum working electrodes. Scale bars = magnification. a = uncolonized electrode after electrochemical cleaning (the platinum surface appears black and the glass surface green); b = electrode covered with a biofilm grown for 1 h; c = electrode covered with a biofilm grown for 7 d.

Several electrochemical experiments were conducted in order to ascertain the behaviour of an uncolonized platinum electrode that was immersed in the growth medium. Figure 4a shows a cyclic voltammogram recorded at an uncolonised platinum electrode, between the potentials − 0.5 and 1.0 V *vs* a Ag/AgCl reference electrode, and it can be observed that there are small differences when compared to the voltammogram depicted in Figure 1 resulting from the change in medium composition.

To find out if the presence of a biofilm on the electrode changed the pattern of the voltammogram obtained compared to the uncolonized electrode, electrochemical experiments were conducted with 3-h biofilms. It is clearly observed (Figure 4b presents the voltammogram obtained for the first scan) that the presence of the biofilm on the electrode surface had a strong effect on the shape and size of the peaks when compared to those observed in Figure 4a.

The shape of the first voltammogram obtained as the potential was cycled may provide information on the coverage of the surface since the areas under the peaks are lower than the peaks obtained for the uncolonised electrode. Moreover, the older the biofilm (as observed in Figure 3, the coverage of the surface increases with the age of the biofilm) the lower are the peaks of the voltammograms, as depicted in Figure 5, which presents the first scan of the cyclic voltammograms obtained for electrodes covered with biofilms grown for 1, 3 and 5 h respectively. This behaviour may be explained by the fact that as the accumulation on the surface

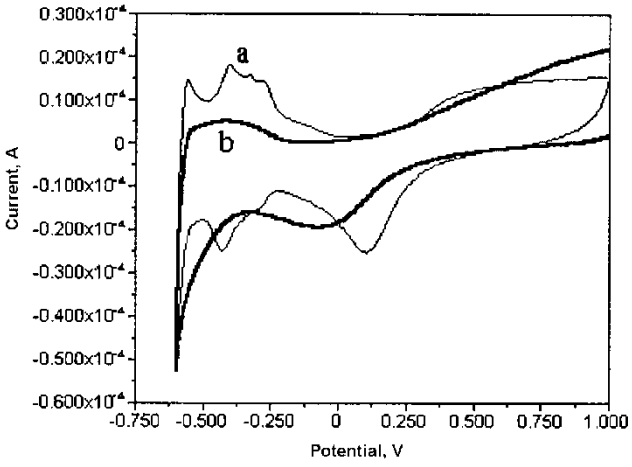

FIGURE 4 Cyclic voltammograms obtained at a platinum electrode in phosphate buffer solution (scan rate 0.25 V s^{-1}, potentials between − 0.5 and 1.0 V *vs* a Ag/AgCl reference electrode). a = uncolonized electrode after electrochemical cleaning; b = electrode covered with a biofilm grown for 3 h.

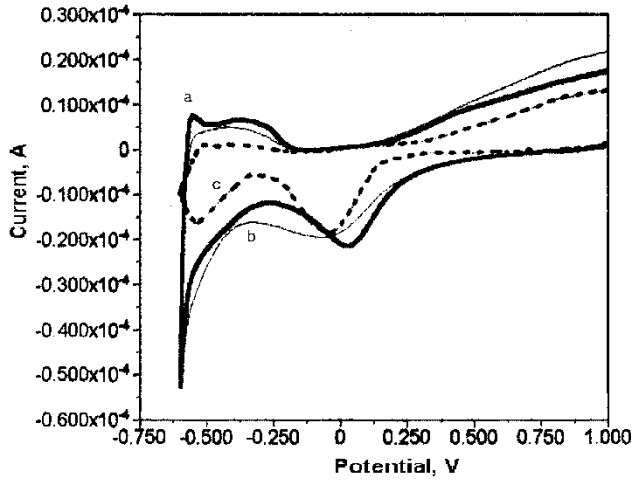

FIGURE 5 Cyclic voltammograms obtained at platinum electrodes in phosphate buffer solution (scan rate 0.25 V s^{-1}, potentials between -0.5 and 1.0 V *vs* a Ag/AgCl reference electrode). a = electrode covered with a biofilm grown for 1 h; b = electrode covered with a biofilm grown for 3 h; c = electrode covered with a biofilm grown for 5 h.

increases, the area available for the oxidation-reduction processes on the surface decreases and, consequently, the area under the voltammogram decreases.

Illsey *et al*. (1997) also used cyclic voltammetry of platinum electrodes to study bacterial adhesion to surfaces over a 4-h period. In this study, where cyclic voltammetry was conducted at 0.5 V s^{-1}, the surface charge density resulting from the adsorbed bacteria was calculated from the integrated current-potential response corresponding to the anodic oxidation in the presence of bacteria and to oxide reduction. The difference between these two charges was considered to be related to the number of adsorbed bacterial cells.

In the current work, the adsorption and desorption of oxygen (peaks 2 and 3) did not give an appropriate measure of the coverage because solutions were not degassed (as occurs in real systems) and therefore, the dissolved oxygen affected the areas under those peaks. The adsorption of the hydrogen peak, on the other hand, was found to be influenced by the reduction of the oxide and would not for this reason be a good choice. Hence, it was concluded that the peak giving the best information about surface coverage was the one corresponding to the oxidation of the adsorbed hydrogen (peak 1). This conclusion was supported by the analysis of cyclic voltammograms obtained at the platinum electrode when immersed in a solution containing 0.5 M sulphuric acid and in the phosphate buffer solution. In both cases the voltammograms were recorded in the presence of dissolved oxygen and after degassing the solutions to investigate the role of oxygen on the shape of the adsorption/desorption peaks.

Effect of Recycling the Potential

Figure 6 presents the effect of recycling the potential between -0.5 and 1.0 V for 30 min on the characteristics of the voltammograms obtained with biofilms that had developed on the platinum electrode for 5 h before being placed in the electrochemical cell. Figure 6a–e shows voltammograms obtained after 1, 2, 5, 10 and 100 potential cycles.

It should be noted that, as the potential is cycled between the pre-set limits, the pattern of the voltammograms approaches that corresponding to an uncolonized surface. As a consequence, this set of data suggests the possibility that the method could be used to achieve complete cleaning of the electrode surface, even when a biofilm has grown on its surface for several hours. However, the observation of the electrode surfaces by epifluorescence microscopy after cycling for 30 min showed that cells were still present on the surface, as shown in Figure 7. This Figure compares the electrode surface with a 2 h biofilm before and after cycling for 30 min.

For very young biofilms, the stabilization of the voltammogram due to recycling is faster than for older biofilms and, in some circumstances may approach that obtained for the uncolonized electrodes. Nevertheless, observation of the electrode surfaces by epifluorescence microscopy after cycling for 150 scans (30 min), demonstrates that, although some of the bacterial cells were removed from the surface, the coverage was still very high, demonstrating that the electrochemical treatment was not able to completely remove the biofilm. As shown in Figure 8, the number of cells remaining on the surface of the platinum electrodes after cycling 1, 3 and 5 h biofilms for 30 min is still very high.

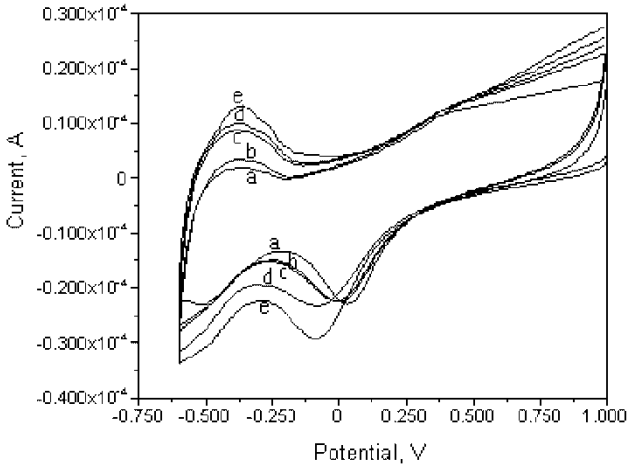

FIGURE 6 Cyclic voltammograms obtained at a platinum electrode in phosphate buffer solution whose surface was covered with biofilm grown for 5 h (scan rate 0.25 V s^{-1}, potentials between -0.5 and 1.0 V *vs* a Ag/AgCl reference electrode). The voltammograms were obtained after 1 scan (a); 2 scans (b); 5 scans (c) ; 10 scans (d); and 100 scans (e).

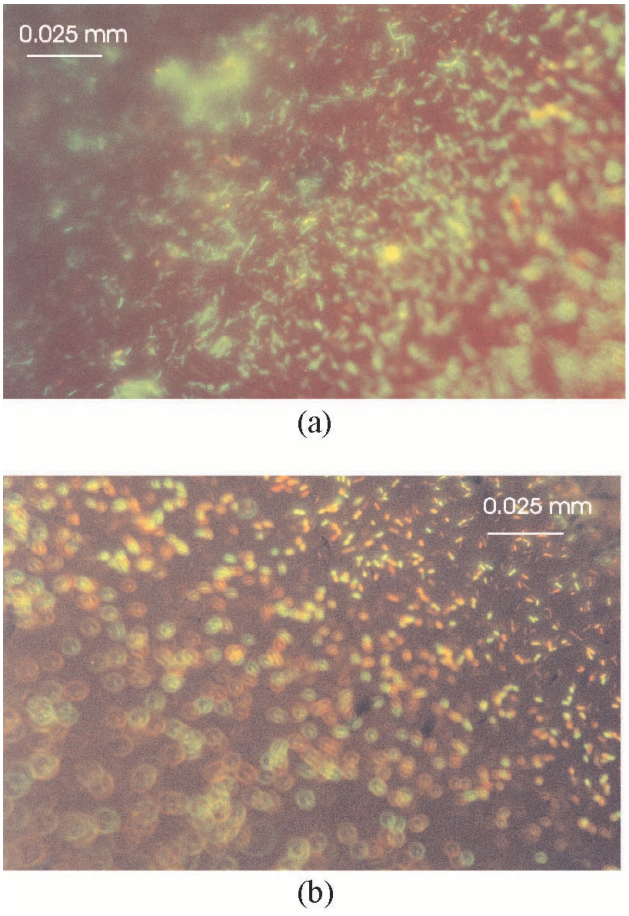

FIGURE 7 Comparison between the epifluorescence microphotographs of the platinum WEs covered with 1-h biofilms before (a) and after (b) the electrochemical measurements (150 scans at a scan rate of 0.25 V s^{-1}, potential limits from − 0.5 to 1.0 V *vs* a Ag/AgCl reference electrode). Scale bars = magnifications.

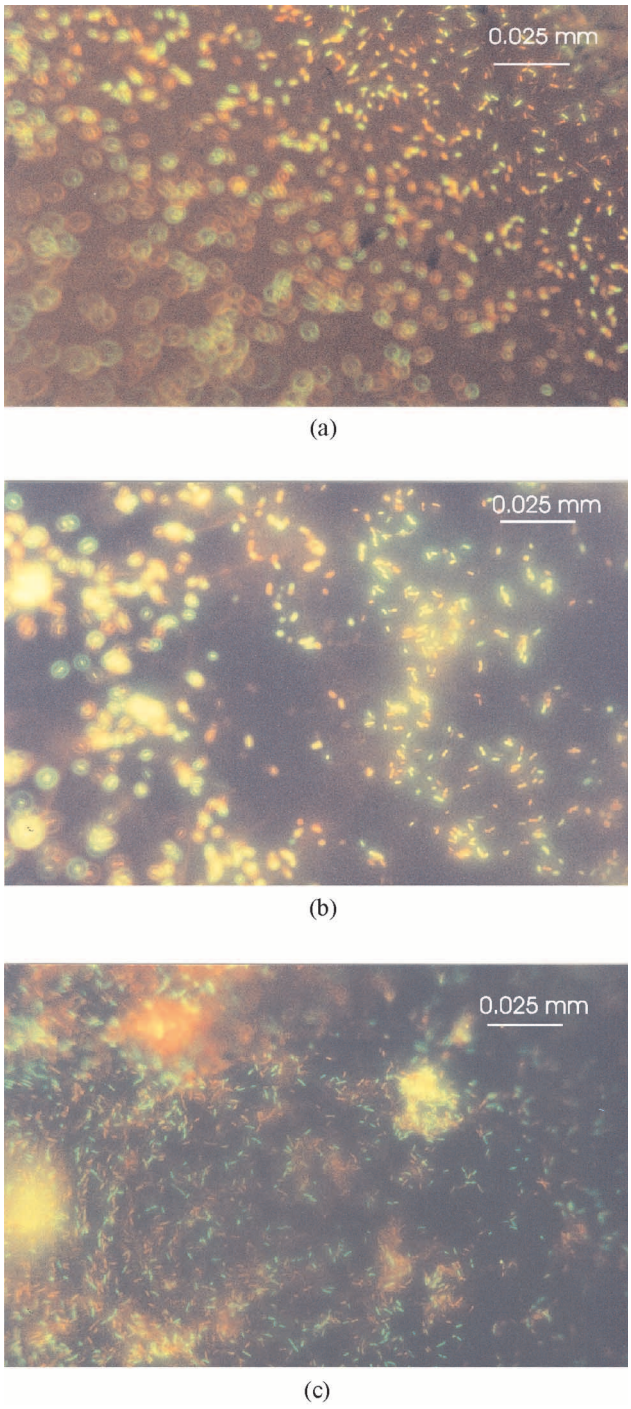

FIGURE 8 Epifluorescence microphotographs of the platinum working electrodes covered with biofilms of different ages after the electrochemical measurements (150 scans at a scan rate of 0.25 V s^{-1}, potential limits from − 0.5 to 1.0 V *vs* a Ag/AgCl reference electrode). Biofilm grown for 1 h (a); 3 h (b); and 5 h (c). Bars = magnifications.

Figures 7 and 8, obtained after staining the biofilm with the Live/dead viability kit, show that some of the cells remaining on the surface after the recycling treatment appear dead (since they are stained red) in contrast to the cells on the surface before the treatment that appear green, demonstrating that they are alive. This result may be associated with the formation of bactericidal H_2O_2 *in situ* (Dhar *et al.*, 1982) or to pH changes at the interface.

The oxidation of the adsorbed hydrogen peak (peak 1) is very sensitive to the age of the biofilm, although when the biofilm was older than 5 h, the pattern of the voltammogram no longer changed. Figure 9 displays cyclic voltammograms corresponding to the recycling of an electrode with a 7-d-old biofilm. This figure shows that the first voltammogram is flat, but after recycling for 30 min the voltammogram approaches that of an uncolonized electrode, in spite of the fact that the biofilm (or part of it) is still present on the surface.

In order to verify the electrochemical behaviour of the platinum electrode after recycling the potential for 150 scans, cyclic voltammetry was carried out again. As shown in Figure 10, the first scan of the second recycling cycle is rather similar to the first scan of the first cycle, indicating, as demonstrated by the photographs, that there are still deposits on the surface.

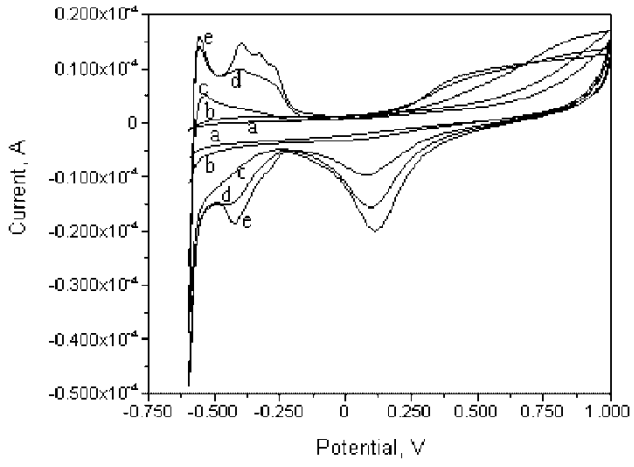

FIGURE 9 Cyclic voltammograms obtained for a platinum electrode in phosphate buffer covered with a 7-d biofilm (scan rate 0.25 V s^{-1}, potentials between -0.5 and 1.0 V *vs* a Ag/AgCl reference electrode). The voltammograms were obtained after 1 scan (a); 2 scans (b) ; 5 scans (c); 10 scans (d); and 100 scans (e).

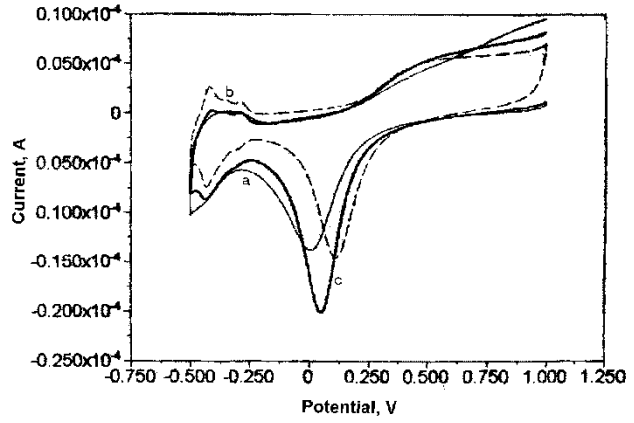

FIGURE 10 Cyclic voltammograms obtained at platinum electrodes in phosphate buffer solution (scan rate 0.25 V s^{-1}, potentials between -0.5 and 1.0 V *vs* a Ag/AgCl reference electrode), for an electrode covered with a biofilm grown for 2 h. a = scan number 1 of the first recycling cycle; b = scan number 150 of the first recycling cycle; c = scan number one of the second recycling cycle.

The possible explanation for the results presented is that for very young biofilms (up to 5 h) the biofilm is constituted mainly of bacterial cells which interact with the surface mainly by electrostatic forces, apart from Lifshitz van der Waals and hydrophobic forces in a situation of irreversible adhesion (the action of removing the electrodes from the fermenter and introducing them in the electrochemical cells removes loosely attached cells). As a result, the distance between the adhered cells and the surface is very low (an interaction on the primary minimum of energy, according to the DLVO theory) so that oxidation-reduction processes cannot occur on some areas of the electrodes, and thus the pattern of the voltammogram is different from that obtained for an uncolonized electrode. As the potential is applied to the platinum surface, the distance between the bacterial cells and the surface may increase (and some bacteria may even be released into the buffer contained in the electrochemical cell) and the liquid film between the bacterial cells and the surface becomes thicker. Consequently, oxidation-reduction processes may occur on the platinum surface previously occupied by cells and a voltammogram similar to that of an uncolonized surface is thereby obtained. After stopping the application of the potential to the surface, the cells may approach the surface again thereby reinstating the initial state of interaction with the surface. As a consequence, the first scan of the second recycling set is very similar to the first scan of the first set; a similar process may occur with older biofilms.

The experiments described above indicate that repetitive cyclic voltammetry applied to a platinum electrode offers a sensitive method to detect biofilms in the early stages of formation. However, platinum is not the material used in industrial flow systems and, therefore, it is important to investigate how the surface properties of the electrode material compare to those of, *e.g.*, SS, which is commonly used in heat exchanger pipes and water distribution systems. This may be conveniently done by measuring the adhesion free energy and the hydrophobicity of both materials (Teixeira & Oliveira, 1999). Although the adhesion free energy is generally negative for most surfaces, *i.e.* the adhesion is thermodynamically unfavourable, the hydrophobicity of the surface favours the deposition of the first layer of bacteria (Oliveira *et al.*, 2001). Table I shows the comparison between such data for platinum and stainless steel. The results indicate that the differences in surface properties between platinum and stainless steel are rather small and, therefore, it can be assumed that the bacteria colonise both materials in a similar fashion.

CONCLUSIONS

The results presented show that cyclic voltammetry applied to platinum electrodes can be used as a means to detect early formed biofilms. The change in the shape of the voltammograms as the potential is cycled may constitute a means of providing information on the coverage of the surface, since the area under the peaks decreases as the coverage of the surface increases. Observation of the platinum

TABLE I. Surface properties of platinum and stainless steel

Material	Adhesion free energy (mJ m^{-2})	Hydrophobicity (mJ m^{-2})
Platinum	43.98	53.02
SS	41.35	45.21

electrodes before and after the electrochemical measurements shows that even after 30 min of recycling, most of the cells continue to be adhered to the surface, although some of them may be dead. Further studies are being carried out to obtain complete electrochemical cleaning of surfaces.

Acknowledgements

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (Fundação para a Ciência e Tecnologia-FCT) through Project POCTI/34945/EQU/2000. The authors are also grateful to Dr Rosário Oliveira and Dr Joana Azeredo (Biological Engineering Department, University of Minho) for determination of the free energy of interaction between the bacteria and the surfaces.

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