Online Monitoring of Electrochemically Active Biofilm Developing Behavior by Using EQCM and ATR/FTIR

Ying Liu^{a*}, Antonio Berná^b, Victor Climent^b, Juan Miguel Feliu^b

^a College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, P.R. China, 712100

^b Instituto de Electroquímica, Universidad de Alicante, Apartado de correos 99, 03080 Alicante, Spain

Abstract

It is crucial to study the bacterial attaching behavior for revealing information of electrochemically active biofilm on the interface of liquid/electrode in microbial fuel cells (MFCs). In the current study, mixed-culture from waste water as model bacteria was investigated for gaining more sight onto relationship between current and biomass or onto bioadhesion mechanism at the molecular level using Electrochemical Quartz Crystal Microbalance (EQCM) and Attenuated Total Reflection-SEIRAS (ATR-SEIRAS) combined with electrochemistry. From the frequency shift using Sauerbrey equation via EQCM the maximum cells mass of $11.5 \mu\text{g}/\text{cm}^2$ was estimated for the mature biofilm. Notably, the highest current density of $110 \mu\text{A}/\mu\text{g}\cdot\text{cm}^2$ occurred before maximum biomass coming, which indicated that mature biofilm may not be an optimal state for enhancing power output of MFCs. Furthermore, the sensed biomass attached on electrode for mature biofilm will be constant for more than 40 h

* Corresponding author, Current address: College of Life Sciences, Northwest A&F University, No. 22 Xinong Road, Yangling, Shaanxi Province, P.R. China, 712100, E-mail: lydiayliu@yahoo.com

even under depletion of substrate, implying that cells attached on surface surrounded by filamentous materials and extracellular polymeric substances (proteins, polysaccharides, humic substances, etc) packed together can tolerant lacking nutrients. On the other hand, using ATR-SEIRAS techniques the obvious adsorbed water structure change during biofilm formation on electrode surface was observed and showed that the absorption bands linked to bacterial adsorption increased. It can be concluded that water adsorption accompanies the adsorbed bacteria and the cells number attaching on the electrode increased with time. Especially, the direct contact of bacteria and electrode via outer-membrane protein can be confirmed via series spectra at amide I and amide II modes and water movement from negative bands displacing by adsorbed bacteria. Our study provided supplementary information about the interaction between the microbes and solid electron acceptors beyond traditional electrochemistry. It is expected to explore more information on electrochemically active microbial kinetics for improvement of their competence and understanding of attaching mechanisms in nature using our approach.

Introduction

Traditional biofilm research based on microbial cell aggregates has long history on studying its bioblocking, biocorrosion, and also on advantages such as treating wastewater using biofilm fluidized bed reactors. A biofilm can be defined as a complex coherent structure of cells and extracellular polysaccharide structures to form spontaneously large and dense granules, or grow/attach on solid surfaces. Recently, biofilms have another remarkable function as biocatalysts in renewable energy of microbial fuel cells (MFCs). The distinct differences of biofilms in MFCs by

comparison to the traditional biofilms are that they are electrochemically active by using the solid electrode as electron acceptor in some step of bacterial metabolism. Electrochemically active biofilm development on electrode surfaces has critical influence on MFC application. At present, much effort has been made to provide new insights into electron transfer processes occurring at the electrode/biofilm interface by combining some modern analytic technologies with traditional methods. The unique technique of scanning tunnel microscope has successfully exhibited the presence of conductive pili nanowires (Gorby et al. 2006; Reguera et al. 2005; Reguera et al. 2006) which have been involved or may play key function in the electron transfer through anode *G. sulfurreducens* with biofilm even more than 50 μm thick (Malvankar et al.). The identification of secreted or metabolized self-mediator or intermediary e.g. phenazine (Hernandez 2004) or flavins in *shewanella* species (Marsili et al. 2008) has been realized by combining electrochemistry, biology, and typical analytical chemistry techniques including high-performance liquid chromatography and chromatography-mass spectrometry, etc. Besides, genetic engineering can identify the complicated outer-membrane system making up of hundreds of cytochromes types (Busalmen et al. 2008; Lovley 2008; Myers and Myers 2000) and find that *c* type of cytochrome in biofilm will be in charge of the electron transfer step. Furthermore, for molecular function group identification, spectroscopy is one of most important methods to give information about molecular structure aspects. In this respect, the interface between *G. sulfurreducens* cells and electrodes was studied using a spectro-electrochemical approach by attenuated total reflection-SEIRAS (ATR-SEIRAS) (Busalmen et al. 2008). Most importantly, Bond group advanced a nondestructive *in situ* linking technology of electrochemical and UV-visible spectroscopy through which the function of redox cytochrome *c* in biofilm *in situ* was

successfully revealed (Liu and Bond 2012a; Liu et al. 2011). These studies strongly confirmed that outer-membrane cytochrome *c* molecules were responsible of the electron transfer processes of biofilms attached on electrodes.

Although these advanced techniques can monitor electrochemical active biofilm growing processes and provide some valuable information, there is still necessary to gain better understanding of the interactions between the microbes and electrodes and find the fundamentals for the improvement of power output in MFCs. It must be understood the advanced synergistic function of cells' metabolism in electrochemically active biofilms using solid electrode as electron acceptors, such as in presence of conductive pili, redox carriers, secreted enzymes, or "wise" arrangement of cells on electrode surfaces, to successfully promote electron transfer of biofilm. These aspects are still in need to be further pursued. Among them, the nearest interface between the biofilm and electrode surface could have plenty of critical information to be revealed. Only if the first layer was developed well and a efficient electron transfer circuit could be built up, then it would be possible to attach the following cells in a stable arrangement. Especially, little has been done to estimate the dependence of electricity production on adherent cells mass. Quartz Crystal Microbalance (QCM) can provide the attached cells as sensed biological element via a piezoelectric mechanism for signal transduction. QCM as a technique to examine interfacial phenomena has been focused on studies of living cells for the process of cellular adhesion for traditional biofilms (Cooper and Singleton 2007; Speight and Cooper) such as osteoblasts (Redepenning et al. 1993), human platelets (Muratsugu et al. 1997), other mammalian cells (Wegener et al. 1998), etc. to reveal normal cell phenotype. However, the QCM crystal was not used as an electrode in most reports. The performance of biofilms producing electricity depends on biofilm mass, biofilm

composition and biofilm structure. It is very important to find the dependence between current generation and biofilm characteristics. The relationship between the proteins of biofilm and current generation was previously reported by using NaOH dissolution and BCA assay and it was found that the current production rate is at 2-8 $\mu\text{A}/\mu\text{g}$ protein in the case of *G. sulfurreducens* (Marsili et al. 2009). While the living cells consisted of protein, lipid, peptidocan, water, polyaccharides, filamentous materials and other relative molecules. All the composition not only protein will contribute to electricity production. Therefore, it is necessary to reveal the relationship between whole cells mass and current generation. Electrochemical Quartz Crystal Microbalance (EQCM) will be sensitive to all attached molecules closely on the quartz surface and was used to nondestructively and *in situ* monitor the formation of biofilms although the study of electrochemically active cells growing at QCM crystal as electron acceptors are still few until now (Brown-Malker et al. 2010; Kleijin et al. 2010; Leino et al. 2011). Brown-Malker (Brown-Malker et al. 2010) showed micro-bioreactor of no more than 100 μL using flow chamber (Brown-Malker et al. 2010; Kleijin et al. 2010; Leino et al. 2011). Especially, using QCM-crystal as electrode working in general bioreactor with volume more than 5 mL was not reported to our knowledge. In our study the usual bioreactor of 15 mL home-made using QCM-crystal as working electrode was used and our results are expected to improve the fundamental understanding of how bacteria interact with insoluble electron acceptors in nature through providing information on microbial kinetics and strength of attachment.

On other hand, ATR-SEIRAS is another powerful technique to investigate the inner layer of exoelectrogenic biofilm (Busalmen et al. 2010; Busalmen et al. 2008) in order to better understand the underlying mechanisms of cellular adhesion. It can study

biofilms, *in situ*, nondestructively, in real time, and under fully hydrated conditions and give real-time feedback on both the spectroscopic and electrochemical response of the working electrode at molecular structure level. As both EQCM and ATR can supply information of the thin layer growth at early stage, here the information about initial attachment and further layer growth of the biofilm will be investigated using *in situ* ATR-SEIRAS spectroscopy, QCM combined with electrochemistry to find the relationship between current, sensed biomass and relative molecular composition. The dependence of current generation on relative mass and molecular information of biofilm will be established. It has the potential to provide a wealth of new data to help optimise the performance of biofilms in these systems.

2. Materials and Methods

2.1. Electrode Materials and Chemicals

Polycrystalline carbon rods (1.5 cm long, 0.3 cm diameter, geometrical surface area 1.48 cm²) were cleaned without polishing. Conductive glue was used to connect the carbon materials with a stainless steel wire, and non-conductive nontoxic epoxy resin covered the junction. All chemicals were of analytical or biochemical grade.

2.2. Electrochemical and Spectroscopic Experiments

QCM922 QCM (Princeton Applied Research, USA) with Au coated 9 MHz AT-cut quartz crystal and electrode working Area of 0.196 cm².

QCM was external connected to the VMP3 multi-channel potentiostat (Bio-Logic, France) with computer controlled data acquisition system. Cyclic voltammetry and chronoamperometry were conducted either on VMP3 potentiostat connected QCM. The electrochemical measurements (half-cell) were carried out in a conventional cell with a three-electrode arrangement, with carbon rod as working electrode (WE1) and QCM-crystal as working electrode (WE2), carbon rod as counter electrodes, using the

Ag/AgCl reference electrode (sat. KCl, 0.198 V vs. the hydrogen standard electrode (SHE)) as reference electrode. Home-built bioreactor with QCM mounted vertically on the side wall of cell of volume of 15 mL in order to avoid cells physically sitting down on the bottom was sealed by rubber lid as shown in Figure 1.

Figure 1 Schematic diagram of bioreactor using QCM-crystal as one of working electrodes to grow biofilm and current and frequency change recorded simultaneously

Spectro-electrochemical experiments were carried out in a special house-made glass vessel containing coated gold film on a nonoriented monocrystalline silicon prism as working electrode just as before (Busalmen et al. 2008). Electrochemical signals were recorded using three-electrode configuration using a bipotentiostat controlled by a universal programmer (model 175, Princeton Applied Research) connected to a PC through an e-corder 401 unit (E-DAQ Pty Ltd.) by combination with ATR-SEIRAS spectroscope. The counter electrode was a coiled gold wire and the reference was an Ag/AgCl reference electrode. The spectra were monitored by ATR-SEIRAS and collected with p-polarized light with a resolution of 4 cm^{-1} and are presented as the ratio $-\log(R_2/R_1)$, where R_2 and R_1 are the reflectance values corresponding to single beam spectra at the sample and reference condition indicated

in the text for each experiment, respectively. A total of 100 interferograms during 43 seconds were recorded for each spectrum.

All experimental operations were anaerobically conducted at room temperature (23 ± 1 °C) and repeated at least five times. All current density values were normalized on the geometric surface area. All chronoamperometry measurements were done under 0.2 V vs. Ag/AgCl reference electrode.

2.3. Medium, inoculum source and enrichment

The mixed-culture from domestic wastewater was inoculated and enriched were same as previously reported (Liu et al. 2008a). In simple words, the electrochemically active bacteria from waste water were colonized under the anode poised at a constant potential of 0.2 V vs. Ag/AgCl reference electrode and the current was controlled by chronoamperometry in batch mode experiments with 150 mL bioreactor containing a 100 mL medium. The substrate and medium replenishment was done regularly until a stable biofilm was formed. Here, we used the biofilm pre-enriched and colonized on electrode as inoculum to form a secondary biofilm. The synthetic wastewater medium was prepared as previously reported (Kim et al. 2005) containing sodium acetate (10 mM, unless otherwise stated) and a nutrient buffer solution at pH 6.85 containing NH_4Cl (0.31 g/l), KCl (0.13 g/l), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (2.69 g/l), Na_2HPO_4 (4.33 g/l), metal (12.5 ml) and vitamin (12.5 ml) solutions (Lovley and Phillips 1988).

2.4. Scanning electron microscope and sample preparation

Biofilm on electrode was fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 mol/L, pH 7.2) for 12 h at 4°C washed three times in buffer supplemented with 1% OsO_4 . Ethanol-preserved specimens were dried in a critical-point drier after dehydration in a graded ethanol series, sputter coated with gold prior to SEM observation, and examined in a Hitachi S-3400N scanning electron microscope (SEM,

Hitachi, Tokyo, Japan) at 15V.

3. Results and Discussion

3.1 Characterization of sensed frequency and current production from biofilm using QCM-crystal as working electrode.

For QCM fundamental oscillation frequency of the piezoelectric quartz crystal is decreased when mass is deposited on it. QCM has a low resolution limit at the nanogram range. The relationship between QCM frequency shift (Δf) and micromass change (Δm) can be described by the Sauerbrey equation (Sauerbrey 1959):

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\mu_q\rho_q}}\Delta m \quad (1)$$

here f_0 the intrinsic resonant frequency (9.00 MHz), Δf the frequency change, Δm the mass change, A the piezoelectrically active crystal area (0.196 cm²), ρ_q the density of quartz (2.65 g/cm³) and μ_q the shear modulus of quartz for AT-cut crystals (2.947×10¹¹ g/cm·s²) which leads to Δm (g) = -1.068×10⁻⁹ Δf (Hz).

Due to the complication in actual biofilm, many factors cannot be taken into account such as viscosity of the solvent with synthesized metabolic compounds accumulation (Chang et al. 2006), viscoelasticity, energy dissipation behavior, even mass from Newtonian fluid condition. Besides, Sauerbrey equation is strictly valid for rigidly attached films (Brown-Malker et al. 2010; Rodahl et al. 1995). Much less value than the actual bound cell mass based on radiolabeled measurement using Sauerbray equation was already reported (Muratsugu et al. 1997). Even the QCM-D only incompletely responded to the massiveness of the bacterial adsorption (Leino et al. 2011). However, Sauerbrey equation still can provide semiquantitative information

about living biofilm formation (Keller et al. 2000). In our study, to simplify the process and factors from such as dissipation energy, viscoelasticity, etc were not considered in the present investigation, which will be further studied compared in the future.

Figure 2(A) Biofilm grown curve recorded as chronoamperometry at 0.2 V (vs. Ag/AgCl) (B) Curve of frequency shift using QCM-crystal as working electrode (mass was estimated from Sauerbrey equation) (C) Dependence of current density versus frequency shift; (D) Relationship of ratio for current to frequency shift versus time during biofilm initial growth after inoculation (data from curves in figure 2A and 2B).

Using Sauerbrey equation, the cell mass attached on the gold surface was estimated from a frequency shift f of 1 Hz corresponding to a mass increase of 0.909 ng. The f decrease implied cells mass increased detected by QCM. From curves in figure 2A and 2B it can be seen that after the secondary electrochemically biofilm was introduced into the bioreactor containing QCM-crystal as working electrode for about 12 h (region I), the current increased to about $3 \mu\text{A}/\text{cm}^2$ and correspondingly, cells mass from 0 increased to about $1.8 \mu\text{g}/\text{cm}^2$. During this period, a steady-state cell

attachment was developing. Over next 12 h (region II) the current increased to about $25 \mu\text{A}/\text{cm}^2$ with a slightly faster speed than before, while a nearly plateau occurred on mass change. In this region, it is possible that more enzymes started to adhere on the surface and/or more extracellular polysaccharides substance (EPS) excreted. The EPS will alter viscoelastic properties of biofilm, which in turn also affects the frequency response (Kleijin et al. 2010). The stable mass in region II together with less than $100 \mu\text{A}/\text{cm}^2$ might result from reversible attaching and rearrangement of the colonies on the electrode surface during the initial attachment. More than 3 times experimental results showed this similar trend. In next another 15 h (region III), the current curve suddenly showed a higher increased slope. This means that the biofilm growth entered into the rapid exponential phase. Accordingly, the attached cells mass increased also much faster than region II. These phenomena showed that current increase is from the graduate accumulation of attached active cells, although as-estimated biomass may be underestimated using the Sauerbrey equation (Muratsugu et al. 1997).

Interestingly, at ca. 40 h in Region III, the increase of current become a little slower and sensed cells mass started to go down. The reason of slower current increase and mass decrease could result from cells' detachment from multi-layer cells. After 68 h in Region IV, the current reached its maximum value of $420 \mu\text{A}/\text{cm}^2$ and then decreased rapidly due to substrate consumption and cells' slow metabolism. By contrast, the sensed cells mass kept going up until 70 h, showing that the increase of adsorption amount for biomass on electrode surface was not inhibited in spite of substrates exhaustion. Till 75 h, the biomass reached its maximum point of $6 \mu\text{g}/\text{cm}^2$ and then decreased slowly again, implying that cells might start to detach from the surface or some attached molecules on the surface desorbed.

The relationship between current and biomass was further analyzed and the results

were shown in figures 2C and 2D. As reported, current increased with mass until about $4 \mu\text{g}/\text{cm}^2$ and then continued to go up while mass changes oscillated between 3.5 and $4 \mu\text{g}/\text{cm}^2$. It can be explained that the initial formation of biofilm attaching on surface can use fimbriae or pili attached irreversibly or adopt physical interaction. Therefore, some weakly attached microbes will be swept away quickly or become reversible attached at the incipiently formed microcolonies. These phenomena may cause slow and irregular frequency change might due to the relative attaching and detaching balance. After initial attachment stage, the formation of a more excreted mixture of a slime-like matrix, termed “EPS”, will aid to stick to the surface and provide strong protection from the surrounding environment disturbance. When reaching the maximum current density of $420 \mu\text{A}/\text{cm}^2$, the current decreased while biomass continued to go up at ca. $6 \mu\text{g}/\text{cm}^2$. The maximum ratio of current to mass at around 60 h showed that $1 \mu\text{g}$ cells mass can produce maximal about $110 \mu\text{A}$ current on QCM-crystal electrode, which is similar with previous result (Kleijin et al. 2010). It should be taken into account that mass measurement is through sensing frequency change of QCM to thin layer on surface not to whole attached cells. Therefore, the cells mass could be underestimated. While current is recorded at a constant potential from many layers of biofilm on the electrode surface. From our previous studies (Liu and Bond 2012b), most cells in biofilm will contribute to the current production although most outer layers may have lower electron transfer efficiency than cells in the deep layers.

3.2 Dependence of current on sensed frequency for mature biofilm in second batch

Figure 3(A) Biofilm growth curve recorded by chronoamperometry at 0.2 V (vs. Ag/AgCl) and (B) Frequency change (cells mass) curve using quartz resonators as working electrode with adding 15 mM acetate after acetate exhaustion in figure 2; (C) Dependence of current density versus frequency shift of biofilm and (D) Dependence of ratio for current to frequency shift versus time

When the substrate was exhausted completely in the first batch, another 15 mM acetate was added in the bioreactor and during chronoamperometry running the frequency was recorded simultaneously at this second batch. Figure 3A and 3B shows the current evolution and the frequency shift as a function of time. Similarly, the mass change was also derived from Sauerbrey equation (1). The current increased for about 15 h till to a maximum value of $530 \mu\text{A}/\text{cm}^2$, which is similar with that on general graphite/carbon or gold electrode with much higher surface area (Liu et al. 2010a; Liu et al. 2008b; Liu et al.). This implied that the biofilm can develop well using QCM-crystal as working electrode. Over the maximum point, the current reduced rapidly below $200 \mu\text{A}/\text{cm}^2$ due to substrate consumption. However the mass decreased in the first 5 hours and then rapidly increased until around 18 h. Afterwards a

maximum steady-state cells attachment of about $11.5 \mu\text{g}/\text{cm}^2$ was kept for more than 40 h. The current showed considerable decrease but mass showed negligible change, which revealing great inhibition from exhausted nutrients on current production and minor influence on cells mass attaching. The sensed mass is possibly from enzyme and/or EPS in cells. The proportion of their synthesized EPS as underlying matrix from QCM surface for sensed cell mass increase can be further studied by comparison of QCM-frequency shift from EPS-producing and non-EPS producing strains using gene-engineered techniques (Rollefson et al. 2011). By comparison with initial and second batches, cell mass was almost 2 times higher than that in the first batch growth of $6 \mu\text{g}/\text{cm}^2$, while the produced maximum current only increased by about one fourth (from 420 to $530 \mu\text{A}/\text{cm}^2$). The maximum sensed mass of $11.5 \mu\text{g}/\text{cm}^2$ by QCM is close to the previous result without considering energy dissipation (Kleijin et al. 2010). Meanwhile, it can be seen that QCM sensing to the attached cells exhibited saturated behavior after two batches' growth processes and nutrient depletion did not obviously influence cells mass response shift on the crystal quartz surface (figure 3B). Maybe communication between cells can supply the nutrients for the growth of inner layer cells or enzymes deeply entrapped in the biofilm matrix might not be greatly affected by starvation.

Curves in Figure 3C and 3D derived from data in figure 3A and 3B showed the direct relationship of current versus frequency shift (mass change) after adding 15 mM acetate. When current increases, the mass decrease (frequency increase) for a short while and then an increasing linear plot dependence from 3 to $9 \mu\text{g}/\text{cm}^2$ was achieved. A maximal current density of $530 \mu\text{A}/\text{cm}^2$ was reached at about $9 \mu\text{g}/\text{cm}^2$. Interestingly, when the current kept going down, the sensed mass still increased slowly. From figure 3D, the current for per microgram cells increased from 40 to 110

$\mu\text{A}/\text{cm}^2$ in the first 5 hours and after that the ratio decreased rapidly again due to current decrease. From figures 2 and 3 together based on Sauerbrey equation, per microgram can produce maximum current of $110 \mu\text{A}/\text{cm}^2$ being almost same in initial and second batch experiments. However, the maximum mass density of $11 \mu\text{g}/\text{cm}^2$ in second batch is nearly 2 times higher than that of $6 \mu\text{g}/\text{cm}^2$ in initial batch. These data showed that effective electrochemical active biomass in biofilm was accumulating with cells growth and closest layers on electrode were robust built in mature biofilm.

On the other hand, the maximum electricity-production efficiency of per microgram happened before maximum current at about 5 h in second batch (figure 3D) not just like in the initial batch (figure 2D) at maximum current point (15 h). Our study reveals that per microgram can produce nearly $110 \mu\text{A}$ current from QCM, which is much higher than that of 2 to $8 \mu\text{A}/\mu\text{g}$ using bicinchoninic acid (BCA) protein assay method (Marsili et al. 2009). The most important factor is that the biomass measured using QCM usually only related to the closest layer molecules of biofilm on gold-quartz crystal surface not the entire cells mass in biofilm, but BCA assay measured all proteins in whole biofilm. Especially, Sauerbrey equation will underestimate biomass because the biofilm is elastic and dissipation energy was not considered (Voinova et al. 1999). Just like Cooper *et al.* suggested that QCM is primarily sensitive to interactions local to the quartz surface and not to multi-cellular layers (Cooper and Singleton 2007). But QCM technique still is attractive for the study of changes in surface adherent cells (1993; Kleijin et al. 2010; Redepenning et al. 1993). Our study provides some information in-depth in certain degree for further exploring biofilm attaching process.

From Figure 4, the thick biofilm growth on QCM-crystal resonator surface was observed after 2 batches' running. The electrochemical active biofilm built well on

QCM-crystal surface was confirmed using SEM images.

Figure 4 Scanning electronic micrograph of mixed-culture growth on QCM-crystal surface

3.3 Bioelectrocatalytic voltammograms and electron redox transfer characteristics of biofilm growth on on QCM-crystal surface

Figure 5(A) Cyclic voltammogram at on QCM-crystal surface with maximum bioelectrochemically catalytic current recorded at 66 h in curve a of figure 2A (scan rate of 5 mV/s). (B) Cyclic voltammogram on QCM-crystal surface with biofilm grown for 4 days at non-turnover status (acetate exhaustion) at scan rate of 1 mV/s, inset showed the cyclic voltammogram before biofilm growth.

In figure 5A, the cyclic voltammogram was recorded at about 66 h with the maximum current in initial batch (figure 2A). As expected, a typical sigmoidal shape with a bioelectrochemically catalytic current of about $500 \mu\text{A}/\text{cm}^2$ was observed. After substrate was exhausted completely, the catalytic sigmoidal shape was changed to a voltammogram with two pairs of obvious redox peaks as shown in figure 5B. By

contrast, no redox peaks was observed before biofilm attached in inset of figure 5B. The redox peaks at formal potential of -0.372 V and -0.289 V resulted from the electrochemically active molecules in biofilm which also were obtained in previous studies (Liu et al. 2005; Liu and Bond 2012a; Liu et al. 2010a; Liu et al. 2008a). Voltammetric features in figures 5A and 5B further confirmed the typical electrochemically active biofilms were successfully developed on QCM-crystal surface *in situ* just like at usual carbon or gold electrodes (Gutman et al.; Liu et al. 2008a; Liu et al. 2010b; Rodahl et al. 1997).

3.4 Time dependent series of ATR-infrared spectra characteristics of biofilm growth on gold surface

Figure 6. Infrared spectrum obtained for the gold electrode surface polarized at 0.2 V (vs. Ag/AgCl reference electrode) in a solution containing culture media in absence of bacteria. The single beam spectrum collected at open circuit potential conditions was used as a reference.

Figure 6 showed infrared spectrum obtained for the gold electrode surface polarized at 0.20 V in a solution containing culture media in absence of bacteria

(before inoculation). The single beam spectrum collected at open circuit potential conditions was used as a reference. During measurement, there is no current observed at open circuit (data not shown). The spectrum suggests that there is a sort of water structure on the electrode with bands at 3300 and 3460 cm^{-1} that would correspond to the symmetrical and asymmetric water stretching, respectively. A band at 1650 cm^{-1} is also observed, that also corresponds to the water bending mode of water adsorbed on the electrode. In addition bipolar bands at 1244 and 1750 cm^{-1} are also observed. These bands increase with the applied potential could be related to anion species related to phosphates present in solution. We should expect that if we do not introduce bacteria, there would be no change in the spectra, only baseline changes (data not shown).

Two single beam spectra from gold film polarization at 0.2 V or at open circuit are going to be taken respectively as reference in the following and exhibit two different behaviors of adsorbed water structure. It will be seen from spectra using beam at 0.2 V as reference that the absorption bands at 1660, 1550, 1400 and 1360 cm^{-1} linked to bacterial adsorption always increase, suggesting that the number of bacteria close to the electrode always increase. These bands correspond to the Amide I and Amide II modes of the external protein membranes of bacteria. By comparison, if the reference spectrum is that measured at open circuit, the time evolution of the spectra always shows positive bands in the 3200-3400 cm^{-1} region, related to water and in the 1200-1800 cm^{-1} , which are related to bacteria (Figure S2 in supporting materials). The positive character of the water bands does not show the real behavior of the adsorbed

water, because these positive bands were also observed when the single beam spectrum at 0.2 V is compared to that taken at open circuit as reference. In this system, the solid electrode was supposed as electron acceptor for the bacteria instead of previous fumarate and solid oxidized iron, while at open circuit potential, the electrode cannot be used as electron acceptor to support bacteria growth.

The first point that should be noted is that there are negative bands in the water region, indicating that water is being displaced by adsorbed bacteria (Figure 7). As observed with *Pseudomonas* (Humbert and Quiles 2011), this water movement points out the direct contact between bacteria and the electrode surface. It is speculated that cells transported to electrode surface via water channels in the biofilm. Interestingly, in addition to the diminution of initially adsorbed water, always evident in the reference spectrum and that yields to the negative band at 3500 cm^{-1} , other water molecules are arriving, i.e. water that accompanies the adsorbed bacteria (bands at $1200\text{-}1800\text{ cm}^{-1}$).

Figure 7. Time dependent series of infrared spectra collected right after the introducing bacteria-electrode and during the subsequent adsorption processes. The spectrum collected with the gold electrode surface polarized at 0.2 V (vs. Ag/AgCl reference electrode) was

taken as a reference. Same conditions as Figure 6. Inset is the current curve corresponding to collecting spectra.

One feature of ATR is that the geometrical range of detection is limited to a thin layer next to the internal reflection element's ($\sim \mu\text{m}$), excluding the monitoring of thick biofilm (ca. $30 \mu\text{m}$). These observations using ATR correlated well with QCM, suggesting an increase of cells mass during biofilm forming.

Conclusion

In this study we demonstrated for the first time using an EQCM to monitor simultaneously the development of the biofilm and current generated by electrochemically active cells on electrode with a normal volume of 15 mL rather than a flow through cell less than $100 \mu\text{L}$. The normal bioreactor not only avoided the possible limitation due to change of medium pH, nutrient concentration or other factors such as pressure in micro-bioreactor and simplified the design but also provided the dependence of biofilm growth on cell mass under more actual conditions. The sensed biomass of biofilm on QCM-crystal surface showed maximum value $6.5 \mu\text{g}/\text{cm}^2$ in initial batch and $11.5 \mu\text{g}/\text{cm}^2$ for mature biofilm in the second batch. The maximum current density is about $110 \mu\text{A}/\mu\text{g} \cdot \text{cm}^2$ in both batches. In addition, the outer membrane attaching in mature biofilm is robust even under starvation. Our results provided some clues about relationship between biomass and current production of closest layer for biofilm formation under applying potential using QCM. On the other hand, the series spectra from ATR-FTIR at amide I and II modes of the external protein membrane of bacteria showed water adsorption accompanied by bacterial displacing on the gold-film surface confirming that the direct contact of

between bacteria and electrode is via outer-membrane protein. ATR-FTIR technique provided a convenient avenue for gaining more insight, at the molecular level, into bioadhesion mechanisms accompanying bacterial adhesion and biofilm development. More information needs to be explored in the further study.

Acknowledgements

This work was supported by project from the National Science Foundation of China (No. 21375107) and University of Alicante through Project CTQ 2006-04071.

Reference

1993. Long-term, on-line monitoring of microbial biofilms using a quartz crystal microbalance. *Anal. Chem.* 65, 65-69.
- Brown-Malker, S., Read, S., Rowlands, A., Cooper-White, J., Keller, J., 2010. Electrochemical Quartz Crystal Microbalance to Monitor Biofilm Growth and Properties During BioElectrochemical System Inoculation and Load Conditions. *ECS Transactions* 28(9), 11-22.
- Busalmen, J.P., Esteve-Nuñez, A., Berná, A., J.M., F., 2010. ATR-SEIRAs characterization of surface redox processes in *G. sulfurreducens*. *Bioelectrochem.* 78(1), 25-29.
- Busalmen, J.P., Esteve-Nunez, A., Berna, A., Feliu, J.M., 2008. C-Type Cytochromes Wire Electricity-Producing Bacteria to Electrodes. *Angew. Chem. Int. Ed.* 47, 4874-4877.
- Chang, K.S., Jang, H.D., Lee, C.F., Lee, Y.G., Yuan, C.J., Lee, S.H., 2006. Series quartz crystal sensor for remote bacteria population monitoring in raw milk via the Internet. *Biosensors & Bioelectronics* 21(8), 1581-1590.
- Cooper, M.A., Singleton, V.T., 2007. A survey of the 2001 to 2005 quartz crystal microbalance biosensor literature: applications of acoustic physics to the analysis of biomolecular interactions. *J. Mol. Recognit.* 20(3), 154-184.
- Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., Culley, D.E., Reed, S.B., Romine, M.F., Saffarini, D.A., Hill, E.A., Shi, L., Elias, D.A., Kennedy, D.W., Pinchuk, G., Watanabe, K., Ishii, S.i., Logan, B., Nealson, K.H., Fredrickson, J.K., 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *PNAS* 103, 11358-11363.
- Gutman, J., Walker, S.L., Freger, V., Herzberg, M., Bacterial Attachment and Viscoelasticity: Physicochemical and Motility Effects Analyzed Using Quartz Crystal Microbalance with Dissipation (QCM-D). *Environ. Sci. Technol.* 47(1), 398-404.
- Hernandez, M.E., Kappler, A., Newman, D.K., 2004. Phenazines and Other Redox-Active Antibiotics Promote Microbial Mineral Reduction. *Appl. Environ. Microbiol.* 70(2), 921-928.
- Humbert, F., Quiles, F., 2011. In-situ study of early stages of biofilm formation under different environmental stresses by ATR-FTIR spectroscopy. *Science against microbial pathogens: communicating current research and technological advances* 2.

- Keller, C.A., Glasmaster, K., Zhdanov, V.P., Kasemo, B., 2000. Formation of supported membranes from vesicles. *Phys. Rev. Lett.* 84(23), 5443-5446.
- Kim, J.R., Min, B., Logan, B.E., 2005. Evaluation of Procedures to Acclimate a Microbial Fuel Cell for Electricity Production. *Appl. Microbiol. Biotechnol.*
- Kleijn, J.M., Lhuillier, Q., Jeremiasse, A.W., 2010. Monitoring the development of a microbial electrolysis cell bioanode using an electrochemical quartz crystal microbalance. *Bioelectrochem.* 79(2), 272-275.
- Leino, T., Raulio, M., Salkinoja-Salonen, M., Stenius, P., Laine, J., 2011. Adsorption of bacteria and polycations on model surfaces of cellulose, hemicellulose and wood extractives studied by QCM-D. *Colloids and Surfaces B-Biointerfaces* 86(1), 131-139.
- Liu, H., Cheng, S., Logan, B.E., 2005. Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. *Environ. Sci. Technol.* 39(2), 658-662.
- Liu, Y., Bond, D.R., 2012a. Long-Distance Electron Transfer by *G. sulfurreducens* Biofilms Results in Accumulation of Reduced c-Type Cytochromes. *ChemSusChem* 5(6), 1047-1053.
- Liu, Y., Bond, D.R., 2012b. Long-Distance Electron Transfer by *G. sulfurreducens* Biofilms Results in Accumulation of Reduced c-Type Cytochromes. *Chemsuchem* 5, 1047-1053.
- Liu, Y., Harnisch, F., Fricke, K., Schroder, U., Climent, V., Feliu, J.M., 2010a. The study of electrochemically active microbial biofilms on different carbon-based anode materials in microbial fuel cells. *Biosensors & Bioelectronics* 25(9), 2167-2171.
- Liu, Y., Harnisch, F., Fricke, K., Sietmann, R., Schröder, U., 2008a. Improvement of the Anodic Bioelectrocatalytic Activity of Mixed Culture Biofilms by a Simple Consecutive Electrochemical Selection Procedure 24, 1012-1017.
- Liu, Y., Harnisch, F., Fricke, K., Sietmann, R., Schroder, U., 2008b. Improvement of the anodic bioelectrocatalytic activity of mixed culture biofilms by a simple consecutive electrochemical selection procedure. *Biosensors & Bioelectronics* 24(4), 1006-1011.
- Liu, Y., Kim, H., Franklin, R., Bond, D.R., Gold line array electrodes increase substrate affinity and current density of electricity-producing *G. sulfurreducens* biofilms. *Energy & Environmental Science* 3(11), 1782-1788.
- Liu, Y., Kim, H., Franklin, R., Bond, D.R., 2010b. Gold line array electrodes increase substrate affinity and current density of electricity-producing *G. sulfurreducens* biofilms. *Energy Environ. Sci.* 3(11), 1782-1788.
- Liu, Y., Kim, H., Franklin, R.R., Bond, D.R., 2011. Linking spectral and electrochemical analysis to monitor c-type cytochrome redox status in living *Geobacter sulfurreducens* biofilms. *ChemPhysChem* 12(12), 2235-2241.
- Lovley, D.R., 2008. The microbe electric: conversion of organic matter to electricity. *Curr. Opin. Biotechnol.* 19(6), 564-571.
- Lovley, D.R., Phillips, E.J.P., 1988. Novel mode of microbial energy metabolism: organic carbon coupled to dissimilatory reduction of iron or manganese. *Appl. Environ. Microbiol.* 54, 1472-1480.
- Malvankar, N.S., Tuominen, M.T., Lovley, D.R., Lack of cytochrome involvement in long-range electron transport through conductive biofilms and nanowires of *Geobacter sulfurreducens*. *Energy Environ. Sci.* 5(9), 8651-8659.
- Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., Bond, D.R., 2008. *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc Natl Acad Sci USA* 105, 3968-3973.
- Marsili, E., Sun, J., Bond, D.R., 2009. Voltammetry and growth physiology of *Geobacter*

sulfurreducens biofilms as a function of growth stage and imposed potential. *Electroanalysis* 22(7), 865-874.

Muratsugu, M., Romaschin, A.D., Thompson, M., 1997. Adhesion of human platelets to collagen detected by Cr-51 labelling and acoustic wave sensor. *Analytica Chimica Acta* 342(1), 23-29.

Myers, J.M., Myers, C.R., 2000. Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *J. Bacteriol.* 182(1), 67-75.

Redepenning, J., Schlesinger, T.K., Mechalke, E.J., Puleo, D.A., Bizios, R., 1993. OSTEOBLAST ATTACHMENT MONITORED WITH A QUARTZ-CRYSTAL MICROBALANCE. *Anal. Chem.* 65(23), 3378-3381.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R., 2005. Extracellular electron transfer via microbial nanowires. *Nature* 435, 1098-1101.

Reguera, G., Nevin, K.P., Nicoll, J.S., Woodard, T.L., Lovley, D.R., 2006. Biofilm and Nanowire Production Leads to Increased Current in *Geobacter sulfurreducens* Fuel Cells. *Appl. Environ. Microbiol.* 72(11), 7345-7348.

Rodahl, M., Höök, F., Fredriksson, C., Keller, C.A., Krozer, A., Brzezinski, P., Voinova, M., Kasemo, B., 1997. Simultaneous frequency and dissipation factor QCM measurements of biomolecular adsorption and cell adhesion. *Faraday Discuss.*, 229-246.

Rodahl, M., Höök, F., Krozer, A., Brzezinski, P., Kasemo, B., 1995. Quartz crystal microbalance set up for frequency and Q-factor measurements in gaseous and liquids environments. *Rev. Sci. Instrum.* 66, 3924-3930.

Rollefson, J.B., Stephen, C.S., Tien, M., Bond, D.R., 2011. Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in *Geobacter sulfurreducens*. *J. Bacteriol.* 193(5), 1023-1233.

Sauerbrey, G., 1959. Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung. *Phys.* 155, 206-222.

Speight, R.E., Cooper, M.A., A Survey of the 2010 Quartz Crystal Microbalance Literature. *J. Mol. Recognit.* 25(9), 451-473.

Voinova, M.V., Rodahl, M., Jonson, M., Kasemo, B., 1999. Viscoelastic acoustic response of layered polymer films at fluid-solid interfaces: continuum mechanics approach. *Phys. Scr.* 59, 391-396.

Wegener, J., Janshoff, A., Galla, H.J., 1998. Cell adhesion monitoring using a quartz crystal microbalance: comparative analysis of different mammalian cell lines. *Eur. Biophys. J. Biophys. Lett.* 28(1), 26-37.