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Real time monitoring of the impedance characteristics of Staphylococcal bacterial biofilm cultures with a modified CDC reactor system

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ABSTRACT

Detection of device-associated infectious processes is still an important clinical challenge. Bacteria grow adhered to the device surfaces creating biofilms that are resistant to antimicrobial agents, increasing mortality and morbidity. Thus there is need of a surgical procedure to remove the indwelling infected device. The elevated cost of these procedures, besides patients discomfort and increased risks, highlights the need to develop more efficient, accurate and rapid detection methods. Biosensors integrated with implantable devices will provide an effective diagnostic tool. *In vivo*, rapid and sensitive detection of bacteria attached to the device surfaces will allow efficient treatments. Impedance spectroscopy technique would be an adequate tool to detect the adherence and the growth of the microorganism by monitoring the impedance characteristics. In this work a label-free interdigitated microelectrode (IDAM) biosensor has been developed to be integrated with implantable devices. Impedance characterization of *Staphylococcus epidermidis* biofilms has been performed achieving electrical monitoring of the bacterial growths in a few hours from the onset of the infection. This pathogen represents the most common microorganism related to intravascular catheters associated infections. The experimental setup presented in this work, a modified CDC biofilm reactor, simulates the natural environment conditions for bacterial biofilm development. The results prove that the low range of frequency is the most suitable setting for monitoring biofilm development. Our findings prove the effectiveness of this technique which shows variations of 59% in the equivalent serial capacitance component of the impedance.

1. Introduction

Microorganisms commonly grow in communities known as biofilms, composed of microbes and a structured extracellular matrix that provides special characteristics to the bio-microsystem (Donlan, 2002; Costerton, 1999). The most important feature of these biofilms is that they exhibit resistance to antimicrobial agents (Davies, 2003). In consequence, bacterial biofilms cause several problems in the industrial and healthcare environments (Costerton, 1999; Hall-Stoodley et al., 2004). Genetic regulation and alterations, physicochemical microenvironments, the quorum sensing, extracellular structures, etc. are some of the processes involved which are still under research. (Davies, 2003; Costerton et al., 1999; Donlan and Costerton, 2002; Prakash et al., 2003).

Many of the current subacute, persistent and chronic infections have their origin in the antimicrobial resistant characteristic of bacterial biofilms (Costerton et al., 1999). It is described in the bibliography that 80% of all clinical cases of infections are related with these biological systems (Williams and Bloebaum, 2010). An elevated number of episodes of nosocomial infections are produced by acquiring the pathogen from the proximal environment, i.e. skin from the patient, or healthcare staff, where the most common microorganism are the *Staphylococcus epidermidis* and *Staphylococcus aureus* (Hall-Stoodley et al., 2004).

It is necessary to point out the relevance of infectious processes associated with indwelling medical devices in the clinical care. These medical implants are the propitious environment for the development of a biofilm after a first colonization. Implantable devices such as urinary catheters, central venous catheters, and mechanical heart valves are potential places to originate an infections process. Approximately 50% of nosocomial infections per year in the US are associated with indwelling devices (Harris and Richards, 2006). In this context the infections not detected in time may end in several medical complications and economical impact.

The detection and the treatment of the infectious processes caused by microbial biofilms are not really solved, even more in

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device-associated infections. The period between finding where the infectious process has been originated and the detection of the symptoms of the patient is often too long (Donlan and Costerton, 2002). The way to face this challenge comes through a better understanding of the biofilm formation progress, in order to effectuate an earlier diagnostic. This knowledge will also provide new therapeutic approaches to biofilm-associated infections (Prakash et al., 2003; del Pozo and Patel, 2007).

A vast development of different techniques has been carried out over the past years for different microbiological applications, especially since biosensors appeared in the research field. These devices present very interesting features for pathogen detection applications, such as low cost, high sensitivity, easy implementation, etc. that makes their selection the better choice for monitoring bacterial growth (Radke and Alocilja, 2004).

One of these techniques is known as impedance microbiology which was first described in the seventies by Ur and Brown (1975) and Cady et al. (1978). It is based on the electrical changes produced as a result of the growth of microorganism, an increase on the number of bacteria and their metabolic activity. Electrical impedance is measured and analyzed under an alternative electrical field through the microelectrodes for a frequency range (Gomez-Sjoberg et al., 2005; Yang and Bashir, 2008).

Interdigitated array microelectrodes sensors (IDAM) are common structures largely used for impedance monitoring (Varshney and Li, 2009). These kind of structures have been tested in combination with different biochemical agents or methodologies, i.e. using magnetic beads combined with antibodies (Yang, 2008; Varshney et al., 2007), immobilized antibodies onto electrodes (Radke and Alocilja, 2005; Tang et al., 2011), and dielectrophoresis effect (DEP) for trapping cells (Suehiro et al., 2003, 2006); or label free biosensors (Yang et al., 2004; Varshney and Li, 2008).

Today, there is a wide variety of novel techniques that allow in-depth *in vitro* studies of the most important aspects of the formation of bacterial biofilms, from gene expression to microscopic characterization. However, there still remains a great demand in the field of *in situ* and *in vivo* detection for indwelling devices due to the clinical and economical impact that it represents.

Unfortunately it is complicated to simulate in the lab the same growing conditions that the natural environment provides. To achieve this challenge some experimental setups have been developed, such as the Center for Disease Control and Prevention (CDC) biofilm reactor, that provides the more realistic, reliable and repetitive conditions for the development of bacterial biofilms expressing their natural phenotypes (Williams and Bloebaum, 2010).

In this work a new monitoring method based on the CDC biofilm reactor system is proposed. This novel setup would help to monitor changes during the evolution of the culture, thus enabling the possibility to identify biomarkers and draw specific treatment strategies. The aim of this work is the characterization of the behavior of biofilm growth and the determination of the parameters that represent better the infected samples inside the modified CDC biofilm reactor.

2. Materials and methods

2.1. Chemical and reagents

The phosphate buffered saline, PBS (0.01 M, pH 7.4), was obtained from Sigma-Aldrich (ref: P5368-10 PAK) and ethanol absolute 99.5% PS was obtained from Panreac (ref: 161086.1121). Saline 4.5% solution (Panreac, PA-ACS-ISO ref: 131659) was prepared with deionized water from Millipore (Mili-Q water). A cleaning 10% dissolution was prepared from Hellmanex II reagent (from Hellma® ref: 9-307-010-507).

The culturing media was prepared in the lab from dehydrated reagents dissolved in Milli-Q water: Tryptic Soy Broth, TSB (BBL™, ref: 211768) enriched with 5% glucose (Dextrose from Difco™, ref: 215530), brain heart infusion, BHI (Bacto™, ref: 237500). All the media were sterilized at 121 °C for 1 h in the autoclave.

2.2. Microbial culture

A strain of *S. epidermidis* was used in this work: the ATCC 35984 obtained from the Colección Española de Cultivos Tipo (CECT). This microorganism was selected due to its high ability to form the slime (Williams and Bloebaum, 2010; McLeod and Sandvik, 2010). All strains were maintained frozen in a Cryoinstant® mixed vial (pH 7.2 ± 0.2, obtained from Scharlau ref: 064-TA8276) and conserved at -80 °C.

The culture was grown from one frozen bead in 2 mL of BHI, and maintained inside the incubator at 37 °C with 10% of CO_2 for 20–24 h. After that time, the vial was washed three times with phosphate buffered saline (PBS) at 4 °C and resuspended in Tryptic Soy Broth (TSB) through vortex and the ultrasonic bath.

Isolation of pure cultures were made from previous broth in harvested plates. A vial of saline was prepared for the strain with isolated colonies of *S. epidermidis* to reach 0.5 McFarland, measured in Densimat equipment from Biomerieux. Two milliliters of the standard preparation ($\sim 1.5 \times 10^8$ bacteria/mL) were inoculated into $\sim$350 mL of TSB inside the vessel of the modified CDC biofilm reactor system at a given time. So the initial concentration of the microbial culture was 8.5×10^5 CFU/mL. The biofilm reactor was placed inside the incubator at 37 °C with 10% of CO_2 over a stir plate set at 150 rpm.

Bacterial concentrations were determined by the standard plating method performing serial dilutions to find the number of microbes/volume. The number of target organisms present in the sample was determined by plating 100 μL of infected media on BHA plates.

2.3. Experimental setup and biosensor

The experimental setup developed for this research is based on the CDC biofilm reactor from Biosurface Technologies (Bozeman, MT). The new patented device (Paredes et al., 2011) consists of a vessel that contains six chip holders with three biosensors in each one making a total of 18 biosensors (see Fig. 1(a)–(c)). These devices are electrically connected to external measurement equipment that provides the electrical signals for measuring. For this work, IDAM biosensors were placed inside the vessel for measuring impedance changes onto their surface, where biofilms are being generated during the microbial development.

The IDAM biosensor was designed and fabricated by means of silicon technologies. A 100 nm thick gold thin film was deposited by RF sputtering process onto 3″ oxidized silicon wafers (see Fig. 1(d)–(f)). The main dimensions of the biosensors are: 7.5 mm width and 14 mm length. The active sensitive surface is a circle of 6 mm in diameter, made up of 118 pairs of 20 μm wide fingers with a separation of 30 μm between them.

Before making the measurements all chips were cleaned with the diluted Hellmanex solution in an ultrasonic bath for 10 min. They were rinsed with deionized water again in ultrasonic bath for other 10 min, and finally another 10 min in ethanol absolute. Once the biosensors were mounted in the chip holder of the modified CDC biofilm reactor, the complete system was sterilized at 121 °C for 1 h in the autoclave.

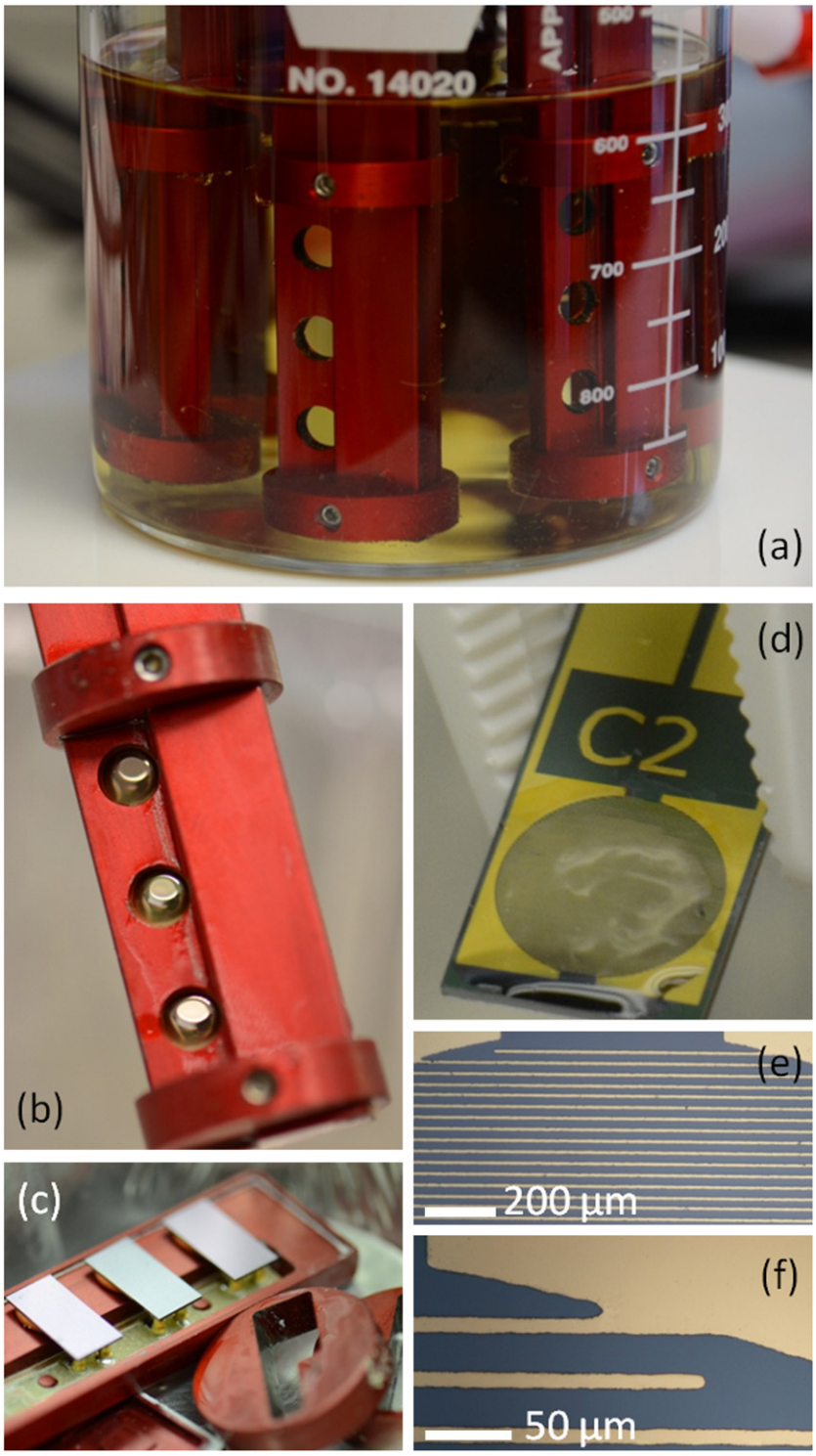

Fig. 1. Real field photographs of the reactor system: (a) the vessel with culture media, (b) the chip holders with three biosensors submerged, and (c) a detail of the internal electrical connections. IDAM biosensor: (d) with an attached bacterial slime onto the surface and (e, f) microphotographs of the microelectrodes acquired with a 4 × lens and a 20 × lens respectively.

2.4. Impedance measurements

Impedance measurements were performed using a *Solartron 1260 Impedance/Gain-phase Analyzer* (*Solartron Analytical*). A multiplexer system, named *MUX4Bio*, has been developed *ad hoc* for this monitoring setup. This device has 24 independent channels operated by relays (Meder ref: BE12-1A74-M) which are controlled by a data acquisition board (DAQ from Keithley Instruments Inc. ref: KUSB-3160). Specific LabVIEW® based software has been developed for controlling the multiplexer through the DAQ and the impedance analyzer. This software also provides real time tracking of the evolution of the impedance measurements for each channel.

Each sensor was measured every 30 min with the same electrical conditions during the 30 h duration of the experiment. A sweep frequency between 100 kHz and 10 Hz was applied to

the samples on the IDAM chips, using constant voltage amplitude mode with a sinusoidal excitation of 50 mV without bias polarization voltage. The measurements were carried out inside the CO_2 incubator system at 37 °C and 10% of CO_2.

3. Results and discussion

3.1. Equivalent electrical model

Electrical impedance is measured onto the biosensors label-free surface between each pair of interdigitated microelectrodes. The electrical performance of the IDAM biosensor submerged in the culturing media could be represented by an equivalent theoretical circuit proposed in Fig. 2. That model shows the electric components that are related to the events that are occurring on the biosensors surface during the represented step times. This circuit is based on previous works of Varshney and Li (2008) and Yang Yang, (2008) where a serial circuit C_{Dl}–R_{sol}–C_{Dl} is presented for data fitting. In a theoretical approximation, R_{sol} represents the electric bulk resistance of the medium distributed above the sensitive surface while C_{Dl} is the double layer capacitance of each pair of microelectrodes (in our model $C_{dl}=2C_{Dl}$) that is also supposed to be distributed.

Before the infection of the sample, (first step in Fig. 2), impedance could be characterized as serial resistance and capacitance for the distributed current lines above the surface of the biosensor (R_{sol0} and C_{dl0}). Different equivalent circuits are reported in bibliography to represent these phenomena that could be also appropriate for monitoring bacterial cultures (Varshney and Li, 2009). Once the medium is infected, impedance starts changing due to two effects: the cells attached to the surface (R_{bi} and C_{bi} are the resistance and capacitance induced by adhered cells) and the metabolic activity of bacteria growing in the media (R_{soli} and C_{dli}). It has to be noted that bacteria will attach itself to all free surfaces in contact to the growth media, so the metabolic activity will belong not only to the bacteria present on the biosensor but also to all microbes growing inside the culturing vessel.

The global measured impedance will change proportionally to the progression of the bacterial growth. The growth kinetics of the bacteria will depend on the nutrients and the flow conditions, so in that variable context, bacteria will behave in different ways, as it is represented in step 5 from Fig. 2: dispersing, persisting, spreading, etc. In such a manner, electrical impedance monitoring is an appropriate tracking tool to determine the growth of microbes.

Many authors are focused on the evaluation of the detection limit or even quantification of bacterial cell concentration of a sample. Their works consist in evaluating the biosensors sensitivity and rapid response to different samples at different microbiological concentrations. For this objective previous cellular suspension has to be prepared from cultured samples to perform the measurements. Thus the detection is performed through bacteria in suspension without taking the natural growth of microbes into consideration.

Emphasis should be placed on the aim of our work, which is the monitoring of bacterial development during the generation of the biofilm. For that purpose an evaluation of biosensors capabilities for implantable *in vivo* applications has been tested. Therefore this point of view is completely different from previous published works, considering that their goals seek in all cases the detection of microorganisms.

3.2. Monitoring of bacterial biofilm cultures in growth media

Fig. 3 shows a Bode plot of experimental data for impedance measurements, both magnitude and phase of one sensor represented at different times. Impedance magnitude and impedance phase curves decrease in time as the culture develops.

As shown in Fig. 3, capacitance behavior dominates at low frequencies ($\sim < 4$ kHz) and the resistive component is the dominant element for high frequencies ($\sim > 70$ kHz). In the intermediate region both R_{sol} and C_{dl} are present in the impedance response showing phase plateau of approximate 60 negative degrees. This behavior agrees with the detection principle presented in the previous section. Impedance magnitude values are in the range of 49 kΩ–25 Ω for 10 Hz–100 kHz respectively, which means that with 50 mV of signal excitation the current values are in the range 1.02 μA–2 mA respectively.

Each one of the 18 biosensors was measured inside the reactor system with the same measurement conditions, assuming that the medium is homogeneous. A sweep frequency has been implemented over the sensors sequentially every 30 min during the test. These packages of measurements lead us to the temporal monitoring of the microbial culture inside the vessel. Fig. 4 represents the mean value of the relative changes of the magnitude of impedance ($|Z|$)

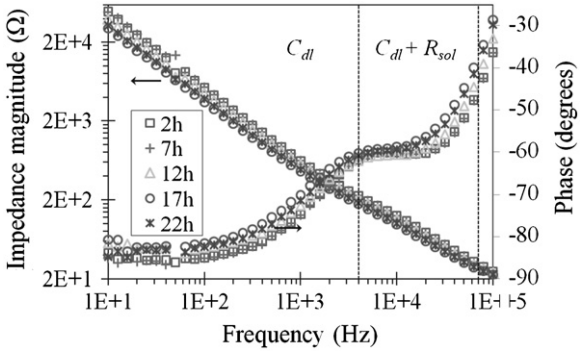

Fig. 3. Bode plot of impedance measurements during *S. epidermidis* growth in BHI media, represented in magnitude and phase of one IDAM sensor recorded at 5 different times.

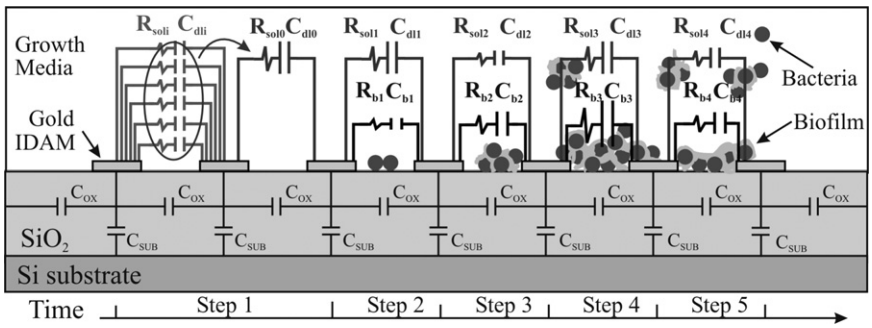

Fig. 2. Cross section of the equivalent circuit model for the impedance measurements of a microbiological culture along different time steps. Initially monitoring starts with clean culturing medium to end with the spreading of the mature biofilm.

and the equivalent serial capacitance (C_{Serial}) of 10 biosensors at different frequencies, from 10 Hz to 100 kHz for the total duration of the trial. Standard deviation error bars are included in alternative selected points as the values remain constant throughout the test.

Microorganisms were inoculated 6 h after the beginning of the monitoring. Previous data were used for establishing a base line as a control of the relative changes. Four hours after the infection impedance starts changing to reach the maximum relative change approximately at 15 h. Those 4 h are related to the lag phase of growth and the attachment process of bacterial cells onto the sensitive surface of the biosensor. Then bacteria move to the exponential phase of growth, where the relative changes become significant.

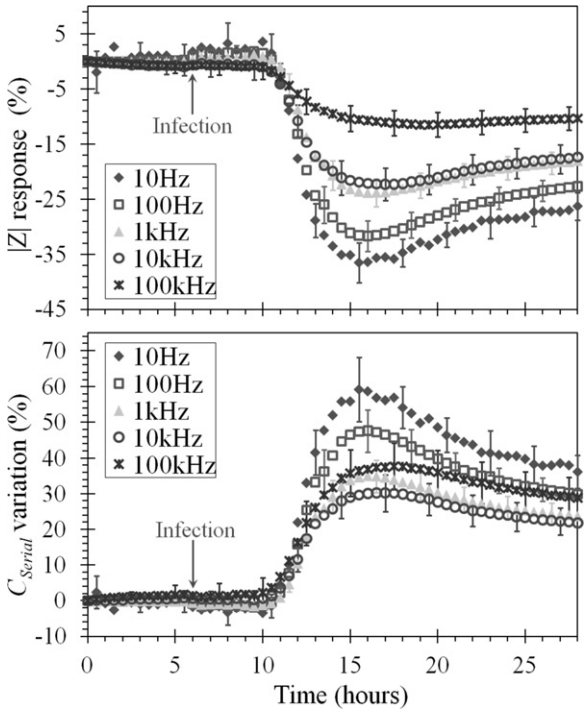

Fig. 4. Relative changes of the magnitude of the electrical impedance and serial capacitance during *S. epidermidis* growth recorded at different frequencies.

Serial resistance component (R_{Serial}) is not represented in the graph because the relative changes in the response are lower that previous parameters. R_{Serial} presents a scattered curve profile at 10 Hz while at 100 Hz presents an increase of 17% of relative variation. Nevertheless the maximum variation takes place at 7 kHz with a decrease of 20% (see Fig. 5). This data agrees with the previous hypothesis where a combination of C_{Serial} and R_{Serial} dominate the impedance response.

Nine hours were needed from the moment of the inoculation of bacteria to reach maximum changes taking into account that the first 4 h for incubation are related to the lag phase of growth and the attachment process. The maximum changes are found in the relative variation of C_{Serial} reaching a 59% and 46% change at 10 Hz and 100 Hz respectively. After reaching the maximum variation of the electrical parameter the curves gradually decrease asymptotically to a saturation state. More likely this effect has to do with the stationary and later death phase of the growth of the microbiological culture.

Curves presented in Fig. 4 are consistent with the experimental data reviewed in the literature where a decrease of the impedance magnitude is associated with an increase of the number of cells in the measured media. Also the development of the culture or the increase of the microorganism concentration is directly related to increased capacitance.

3.3. Effect of frequency on the impedance measurements

Fig. 5 presents the mean of the relative variations of the electrical parameters, $|Z|$, θ, C_{Serial} and R_{Serial}, along the frequency range for 5 different times, 2, 7, 12, 17 and 22 h. The two hour data is taken as the reference value for the relative calculations.

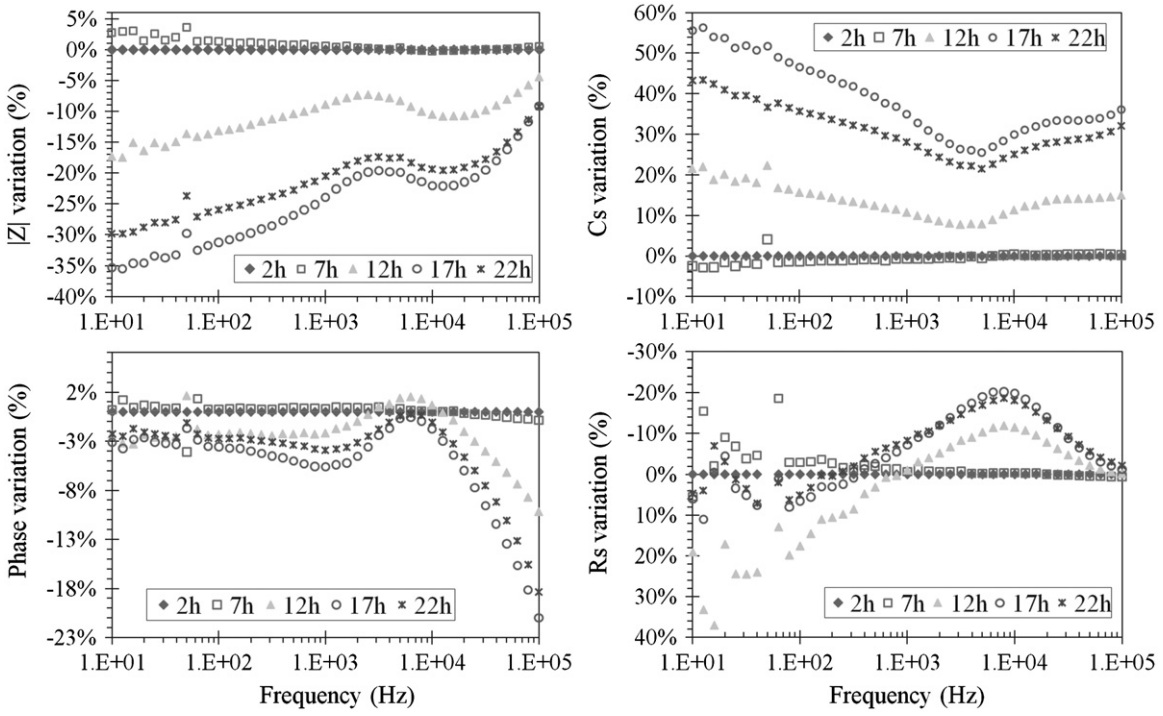

Fig. 5. Relative variation of the electrical parameters of the impedance measurements along the frequency range recorded at different times.

Table 1
Summary of some relevant works of pathogen detection using interdigitated microelectrode biosensors sorted by the detection principle.

Detection principle	Microbe	CFU/mL	Vol.	Detect. time	Freq.	Rel. change	Reference
Label-free Impedance	*Salmonella Typhimurium*	4.8×10–5.4×10^5	5 mL	10–2h	10 Hz	~30%	Yang et al. (2004)
	E. coli O157:H7	8×10–8×10^8	400 pL	14.7–0.8 h	1 MHz	5%–30%	Varshney and Li (2008)
	E. coli	1×10^{10}	2.5 mL	12 h	20 Hz–100 kHz	12%–6%	Zikmund et al. (2010)
Antibody-coated magnetic beads	*Salmonella Typhimurium*	3.45×10^7	25 μL	25 min	1 kHz	22.50%	Yang (2008)
	E. coli O157:H7	8.4×10^2–8.4×10^8	60 nL	35 min	16 kHz	14%–82%	Varshney et al. (2007)
	E. coli O157:H7	7.4×10^1–7.4×10^7	2 μL	35 min	40 kHz	15%–51%	Varshney and Li (2007)
Antibody coated sensor	*S. aureus*	4×10^7	100 μL	10 min	1 kHz	~66.6%	Tang et al. (2011)
	E. coli O157:H7	1×10^4	1 mL	10 min	1 kHz	~280%	Radke and Alocilja (2005)
	E. coli O157:H7	1×10^4	1 mL	35 min	1 kHz	~104%	Radke and Alocilja (2004)

Curves at 7 h present no difference with their reference curve whereas the curves at 12, 17 or 22 h, have significant changes.

Throughout the frequency range analyzed, the variation of the serial capacitance (ΔC_{Serial}) shows change variations between 30% and 59% at 17 h. It supposes that variations at 10 Hz double the variations at 50 kHz. In the same way, the magnitude variations ($\Delta|Z|$) present variations from 35% to 15% at the same frequencies that again are double.

This data together with the standard deviation values, highlight that the best range to monitor the development of the culture is the lowest frequency (<1 kHz). In contrast, the lower the frequency the higher the noise in the measurement, therefore, it is necessary to reach a compromise between the variation and the noise of the measurements. Yang et al. (2004) have also suggested that the low frequency is the suitable range to monitor impedance changes as published in their work.

The values of the impedance phase remain constant below approximately 50 kHz, as a result of the stable capacitive behavior at those frequencies. But above 50 kHz the phase undergoes a major change, so the impedance behavior becomes more resistive; the phase gets closer to 0°. That effect could be associated with the change of the conductivity of the media as a consequence of the metabolic activity of the microbes.

We could compare this data with the results achieved by Kim et al. where they presented a 15% relative change in capacitance component. The difference is that they used a CDC biofilm reactor system only for the generation of the *Pseudomonas* biofilm over different platinum coated disks. Then the disks are placed inside a specific electrochemical cell as electrodes for the subsequent impedance measurement achieving single time data. In this sense all variations recorded by Kim et al. are associated with the attached biofilm and not with the metabolic activity of the bacteria. Finally, after 20 h of incubation, they recorded a saturated signal at the maximum relative variation of 15% (Kim et al., 2008).

Some authors as Ben-Yoav et al. (2011) or Bayoudh et al. (2008) simulate the biofilm formation process inside an impedance spectroscopy-based flow chamber by pumping bacterial suspension that will attach to the electrodes. They analyze the continuous accumulation process by monitoring the impedance changes during time. In the first case, results show a ~50% variation of impedance through the first 10 h of incubation of *Escherichia. Coli*. In the second case, cultures of *S. epidermidis* and *P. stutzeri* leave a ~10% variation of impedance magnitude over 120 min. Both works are based on the principle that the bacterial biofilm was formed as a result of the aggregation of the cells.

Another approach to this challenge was proposed in the work of Zikmund et al. (2010)where monitoring of the growth of *E. coli* was performed over interdigitated microelectrode biosensors. In a similar way the authors simulated the biofilm growth by the sedimentation of bacteria on the biosensors through gravitation effect. Their results show a ~12% variation of relative impedance change of a 12 h culture at 20–100 kHz.

There is also great interest in the evaluation of the detection limit or even the quantification of bacterial cell concentration of a sample for some certain applications. Thus some publications present the performed measurement on bacteria in suspension without regarding the natural growth of microbes as biofilm. The main goal of those works is the evaluation of the sensitivity and rapid response of the method for different microbiological concentrations.

A summary of some of the relevant works on the detection of bacterial samples with interdigitated microelectrode biosensors is presented in Table 1 as a new comparison of our method. Information is organized according to the detection principle of the biosensor. Clearly the most analyzed pathogen was *E. coli* due to the relevant role that it plays in food industry applications. The three methods based on interdigitated microelectrode biosensors: label-free, magnetic-coated beads and antibodies coated method are clearly distinguished in this table.

The first group is related to label-free impedance sensors, which means that no bio-recognition element has been used. The presented relative changes in measurements are in the range of 5–30% of variation for initial concentrations of inoculated microorganisms comparable to those used in our study, as well as the detection times. However there are some differences that are related to the unique performance of the experimental procedure in each case.

Expectation of the response of biosensors combined with immunoseparation or with magnetic nanoparticles, is to be more sensitive and selective than the label-free biosensors in such a less time. As shown in Table 1 the relative variations of the measured parameter are significantly higher than the first group. The detection time never exceeds 35 min regardless of the time prior to sample preparation. These solutions, however, could not be easily implemented for the integration with long time implantable devices.

4. Conclusions

A new method based on a CDC biofilm reactor system was successfully implemented creating a reliable and repeatable environment to generate and monitor bacterial biofilms development. The presented experimental setup favors realistic conditions to characterize both biosensors and biofilm growth with special relevance for *in vitro* and *in vivo* applications.

Label-free impedance biosensors were used for direct monitoring of the *S. epidermidis* bacterial biofilm development in vitro, where bacteria were cultured in a propitious environment that favors the biofilm formation. Interdigitated microelectrode sensors were tested to exhibit suitable capacitive behavior and stability. Relative variations of 59% were measured in the equivalent serial capacitance at 9 h from the beginning of incubation of an 8.5×10^5 CFU/mL initial inoculum sample.

S. epidermidis is a relevant pathogen in device-associated infections that causes significant medical complications and economical costs. The implementation of real time impedance monitoring of indwelling device surfaces will provide new prevention strategies for infectious processes. Impedance technique was proved to be an easy and effective analysis method which can be implemented in any kind of implantable device.

In this context, the low frequency range (10–100 Hz) is the most suitable range to analyze the biofilm attachment to the biosensor surface. At higher frequency ranges the influence of the culture media is more significant; which makes it the most adequate to analyze the metabolic activity of the bacterial community. Specifically, a measurement range around 100 Hz is suggested in order to obtain a proper signal to noise ratio.

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