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# Simultaneous monitoring of *Staphylococcus aureus* growth in a multi-parametric microfluidic platform using microscopy and impedance spectroscopy

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ABSTRACT

We describe the design, construction, and characterization of a scalable microfluidic platform that allows continuous monitoring of biofilm proliferation under shear stress conditions. Compared to other previous end-point assay studies, our platform offers the advantages of integration into multiple environments allowing simultaneous optical microscopy and impedance spectroscopy measurements. In this work we report a multi-parametric sensor that can monitor the growth and activity of a biofilm. This was possible by combining two interdigitated microelectrodes (IDuEs), and punctual electrodes to measure dissolved oxygen, K+, Na+ and pH. The IDuE has been optimized to permit sensitive and reliable impedance monitoring of *Staphylococcus aureus* V329 growth with two- and four-electrode measurements. We distinguished structural and morphological changes on intact cellular specimens using four-electrode data modeling. We also detected antibiotic mediated effects using impedance. Results were confirmed by scanning electrode microscopy and fluorescence microscopy after live/dead cell staining. The bacitracin mediated effects detected with impedance prove that the approach described can be used for guiding the development of novel anti-biofilm agents to better address bacterial infection.



## 1. Introduction

Bacterial attachment and the development of microbial communities commonly known as biofilms cause equipment damage and product contamination and are prominent sources of infection [1]. For example, in the clinical setting, the infection on implants and/or indwelling medical devices such as catheters or heart stents requires a complete removal of the device from the patient very often [2]. In this regard, the attention towards the development of novel lab on a chip platforms to address bacterial infection has increased in the past years [3–5]. These platforms are very attractive because they allow the continuous flow of nutrients. Such conditions are more realistic regarding pathogenesis. Moreover, lab on a chip platforms offer the possibility to study biofilm formation under controlled conditions. Biofilm formation has been described in the literature as a three-step process [6]. It starts with an initial attachment of the bacteria to the material surface by the action of physical forces followed by the formation and maturation of a strong bacterial layer by secreting extracellular polymeric substances (EPS) that provides the biofilm a high resistance to antibiotics [7,8].

To study this biofilm formation methods of continuous label-free monitoring techniques such as surface plasmon resonance [9,10] and quartz crystal microbalance [11] have been developed in the lasts years also gaining a lot of attention in microbiology. The quartz crystal microbalance is able to calculate the amount of biomass attached on the surface applying mathematical approximations. Although both techniques have shown promising results, they are not designed for in-field analysis as they require skilled personnel and the use of equipment that is expensive, non-portable, and cumbersome to assemble. Optical microscopy is a cheap and simple alternative method for characterizing biofilms but, at the same time, it is an end-point assay that involves labeling and destruction of bacteria. Therefore, it is pertinent to develop tools that allow non-disruptive, continuous and label-free monitoring of the dynamic processes and mechanisms of biofilm formation under real conditions [12].

A widely used alternative method for microorganism growth detection is based on electrical impedance spectroscopy measurements [13]. Numerous *in vitro* studies can be found in the literature based on the

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relative impedance changes induced after cell adhesion onto the electrodes [14,15], some of them using commercial interdigitated microelectrodes (IDuE) [16,17]. Despite offering a large sensitive area in a limited space, most of commercial IDuE do not offer the possibility of studying cell growth under flow conditions, a variable that is known to affect biofilm structure and behavior [18], or to measure additional cell culture parameters to control other cell cultures analytes of interest [19]. Furthermore, not many studies have been published on biofilm growth monitoring in a microfluidic channel using IDuEs [20–23], a technology that deserves further study and refinement.

To address some of the technological limitations faced by the conventional electrodes and current devices for bacterial biofilm growth primarily based on cultivation within multi-well platforms, we have designed and fabricated our own multi-parametric sensor and low-cost microfluidic platform. The system is designed to be used in multiple environments and to allow simultaneous and continuous monitoring of biofilm proliferation under shear stress using optical microscopy and multiple read-outs. The chip used in this study contains two IDuEs (rectangular and circular) and punctual electrodes to measure dissolved oxygen (DO) [24], K+, Na+ and pH. Two- and four-electrode configuration impedance measurements can be performed indistinctly on the same IDuE. In this work we optimize the rectangular IDuE geometry with a finite element model (FEM) to reduce the measurement spatial resolution while maintaining a large electrode area. The culture area of the proposed platform is fabricated using transparent material including the electrode substrate to enable the comparison with microscopy evaluation techniques. Real-time monitoring of the important pathogen, *Staphylococcus aureus*, biofilm formation during 24-h experiments using the optimized IDuE was compared to a circular IDuE electrode similar to that from Roche Applied Science (Basel, Switzerland). Susceptibility to the antimicrobial peptide bacitracin was also investigated. The results were confirmed by scanning electrode microscopy and fluorescence microscopy after live/dead cell staining of the bacteria in the measured biofilm.

## 2. Materials and methods

### 2.1. Multi-parametric sensor

This section details the overall design of the multi-parametric sensor. In this work, we have focused only on the performance of the impedance characteristics. To optimize the physical dimensions and geometry of the IDuE (Fig. 1A), we simulated a 2D cross-section FEM model of the electrode geometry with a mesh of 20,000 elements (Fig. 1B) with COMSOL Multiphysics® 4.4 (Burlington, MA, USA). The impedance sensitivity, which depends on the electrode dimensions, was calculated according to [25]

$$S = \frac{\vec{J}_1 \vec{J}_2}{I^2}, \quad (1)$$

where $S$ is the sensitivity to the conductivity changes as a function of the position, $J_1$ is the current density vector when the current is injected between I+ and I− electrodes, and $J_2$ is the current density vector when the same current is injected between the voltage sensing electrodes V+ and V−.

The results in Fig. 1B reveal that the area where the IDuE sensitivity is higher corresponds to gap area between the IDuE groups denoted in the figure as $W_{INT}$. The FEM results (Supplementary Fig. 1) reveal that the optimal geometry is a trade-off between minimizing $W_{INT}$ while also keeping minimal $W_D$ and $n_D$, and maximal $L_D$. In all, the dimensions of the IDuE are $W_{INT} = 21$ μm the interdigitated group space, $W_D = 21$ μm the digit width, $n_D = 32$ the number of digit pairs and $L_D = 7.11$ mm the digit length (detail shown in Fig. 1C). The chip dimensions are 23 mm × 18 mm.

The chip also incorporates additional sensors (Fig. 1D) described elsewhere [24,26] for measuring different biofilm parameters of interest. The punctual electrodes allow to make electrochemical measurements of DO, K+, Na+ and pH. The amperometric measurement of DO is achieved using a working electrode (WE), a reference electrode (RE) and a counter electrode (CE) [24]. The measurement of Na+ and K+ ions is performed with a potentiometric ion-selective technique using the WE for each ion measurement and the RE [27]. Finally, the pH sensor consists of a punctual electrode with an iridium oxide layer using the WE and the RE [26].

The chip was fabricated in the clean room facilities at the Barcelona Microelectronics Institute, Spain, through standard photolithography techniques [28–30]. Three different metal layers (titanium, nickel and gold) were deposited by sputtering over a 500 μm thick Pyrex wafer. First, a 15 nm titanium layer was placed just to improve the adhesion of subsequent metals. Then, a 15 nm nickel layer was deposited on top to the Ti layer to provide a diffusional barrier and to prevent the formation of intermetallic titanium–gold compounds. Finally, the active metal layer was obtained by depositing 150 nm of gold. Thereupon, the electrodes and the metal tracks were patterned using selective wet etching baths, according to a standard lithographic process. At the end, a double layer of 400 nm of silicon oxide and 400 nm of silicon nitride (Si3N4) was deposited by plasma enhanced vapor deposition. This double layer was used for passivation and defining the electrode active area and the connection pads. After the clean room process, a disk saw was used to dice the wafer into individual chips (8 per wafer). Finally, each chip was characterized by cyclic voltammetry using saline solution (0.9% sodium chloride) to verify that there was no shortcut between the IDuE fingers.

### 2.2. Microfluidic device design and fabrication

Initially, the design of the flow chamber was based on the general microfluidic description reported in [31]. Although the general trend in the field is to reduce both the channel and the chamber size [32], in our approach, the minimal dimensions were constrained by the requirement to allow visual inspection and time-lapse recording of the experiment with a microscope. The final size of the platform is 60 mm × 43 mm (Fig. 2), sufficient to accommodate the microscope objective. Since it has been reported that the biofilm attaches to (non-)biological biocompatible surfaces [33], the device is made of a combination of polycarbonate and polydimethylsiloxane (PDMS).

Polycarbonate is used for the construction of the upper and lower lids (Fig. 2A). The top lid holds the connectors, the screws, and the spring-loads for the electrical connections to the sensor. The bottom part is a solid support containing the PDMS support for the sensor and the threaded holes to close the device in a single piece (Fig. 2B). The platform was designed using Catia V5R19 and built with 3D printing technology with fused deposition modeling (FDM) technology. The microfluidic channel and the sensor support were made of PDMS because of its characteristics: biocompatibility [34], gas permeability to supplement oxygen while the nutrients were pumped through the channel, mechanical flexibility to avoid chip fracture and, finally, optical transparency [35] to allow microscopy imaging over the sensing area. The parts fabricated with PDMS (Fig. 2C) were patterned using a replica molding technique applying a master structure made of the negative piece printed in 3D with FDM technology as well. Once the device is closed, the bacterial culture is only in contact with PDMS and the biosensor (Fig. 2D).

The use of the spring-load connectors provided a good electrical contact with the pads and simplified the process of replacing the chip when necessary. It also made possible to reuse the sensor by autoclaving. Besides, the biofilm formed could be imaged easily with a confocal microscope because the sensor, as an independent part, fitted perfectly with the cover slip to perform an end-point assay.