

# Biofilm Oxygen Profiling using an Array of Microelectrodes on a Microfabricated Needle

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## Abstract

A novel microelectrode array (DO-MEA) sensor was designed and fabricated using microelectromechanical systems technology on a needle for real time measurement of dissolved oxygen (DO). The sensor consisted of eleven gold disk microelectrodes and a rectangular auxiliary electrode along them. The sensor can also be operated with an external reference system. Three different sensor designs were fabricated, and their responses were fully characterized and evaluated under different environmental conditions. The DO-MEA sensor presented a linear response in the 0-8 mg DO-l-l concentration range in water, displaying high sensitivity and repeatability, and low detection and quantification limits, below 0.11 mg DO-l-l and 0.38 mg DO-l-l respectively. Knowledge of bacterial activity inside biofilms is key to the optimization of applied biotechnologies. The developed sensor was validated against a commercial Clark-type microelectrode overcoming its drawbacks, by profiling a heterotrophic biofilm cultivated in a flat-plate bioreactor. The DO-MEA sensor provided a multipoint, simultaneous dissolved oxygen snapshot profile inside a biofilm with high spatial resolution due to its micrometric dimensions, thus becoming a powerful tool for the research of many similar biological-based processes and applications.

*Keywords:* Dissolved Oxygen; Microelectrode Array; Microsensors; Bioprocess Monitoring; Profile; Biofilms

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## 1. Introduction

Biofilm formation affects most waste-water and waste-gaseous treatment processes. Both treatment bioreactors use biofilms to remove pollutants [1], for this reason it is critical in bio-reactor design to understand the phenomena and mechanisms governing biofilm growth dynamics. Among the many chemical species that can be found and monitored inside biofilms, oxygen is perhaps the most important, as it is the primary electron acceptor in most aerobic biological processes. When characterizing biofilms, microsensors are powerful tools for the determination of chemical parameters with a high spatial resolution. In the measurement of dissolved oxygen, Clark-type microsensors are the most commonly used probes [2]. However, their manufacture presents important limitations [3]. Most of these microsensors are hand crafted from pulled glass capillaries, and so their main limitations are the fragility and high cost per unit, the tip-size variability between different probes, and the difficulty to bundle several microelectrodes in arrays of controlled geometry. Microfabrication techniques allow the cost-effective mass-production of microsensors that can be used in a vast number of applications [4-5]. This work presents the development of a novel DO-MEA sensor to measure DO profiles which overcomes the limitations presented by Clark-type microsensors by profiling the levels of DO in an heterotrophic biofilm cultivated in a flat-plate bioreactor (FPB) with simultaneous, real time multipoint measurements.

## 2. DO-MEA fabrication and evaluation

### 2.1. DO-MEA Sensor fabrication and preparation

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The novel DO-MEA was fabricated through standard photolithography techniques as it is well reported in the literature [6]. In summary, three metal layers were deposited by sputtering over a 500  $\mu\text{m}$  thick Pyrex wafer. A thin titanium layer (15 nm) was deposited first to improve the adhesion of subsequent metals, a second nickel layer (15 nm) was deposited in order to provide a diffusional barrier and prevent the formation of intermetallic Ti-Au compounds, and a final gold layer (150 nm) was deposited. Subsequently, electrodes and metal tracks were patterned using selective wet etching baths following a standard lithographic process. Finally, SU-8 negative photoresist was chosen as the passivation material due to its optimal dielectric properties and ease of implementation [7]. The microsensor design consists of an array of eleven gold disk microelectrodes (WE), with an integrated macroelectrode that can be used as a counter electrode (CE) and one disk microelectrode that can be used as a reference electrode (RE). Due to the wide range of biofilm thicknesses, three different DO-MEA sensor designs (WE diameter of 50, 25 and 10  $\mu\text{m}$ ) have been manufactured and characterized. Fig 1. shows the different designs.

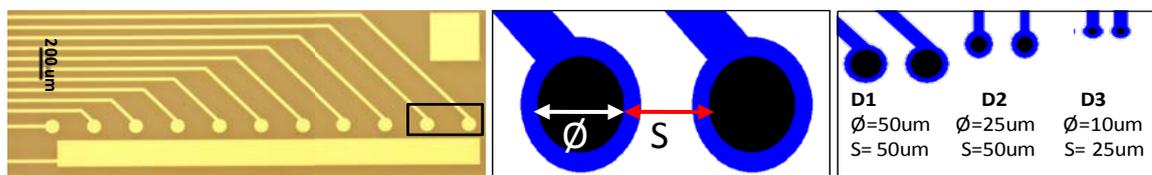


Fig.1 DO-MEA design of 11 gold disk microelectrode array and a rectangular macroelectrode on a 500  $\mu\text{m}$  thick Pyrex substrate and featuring SU-8 passivation.

In order to prepare the microelectrodes surface, its activation is required before use. Several known gold-cleaning methods were investigated [8]. Immersion of the sensor for 1 hour in a solution of 75% v/v 50mM KOH and 25% v/v of  $\text{H}_2\text{O}_2$  was the selected method to clean the DO-MEA sensors. Before and after the cleaning procedure, each electrode was subjected to a cyclic voltammetry from 500mV to -200 mV, at a scan rate of 100  $\text{mV}\cdot\text{s}^{-1}$  in 0.01M ferro/ferri cyanide and 0.1M  $\text{KNO}_3$  solution to determine its state of activation.

## 2.2. DO-MEA sensor characterization and calibration

Sensors were calibrated in the oxygen concentration range between 0 and 8  $\text{mg DO}\cdot\text{L}^{-1}$  range. Oxygen was measured amperometrically using the set-up depicted in Fig. 2a. The sensors were polarized at -850 mV vs Ag/AgCl (3M KCl) as the optimal potential values for the determination of the DO concentration, that involves the complete  $4e^-$  reduction of oxygen achieving higher sensitivity. The DO concentration was adjusted bubbling different nitrogen ( $\text{O}_2$  free) - air (21 %  $\text{O}_2$ ) mixtures through a 0.1 M  $\text{KNO}_3$  solution, and a magnetic stirrer was used to ensure better mixing of the solution. The concentration of DO in the cell was measured with a commercial DO probe and correlated with the measured polarization currents of each gold microelectrode in order to build the calibration curves shown in Fig 2b.

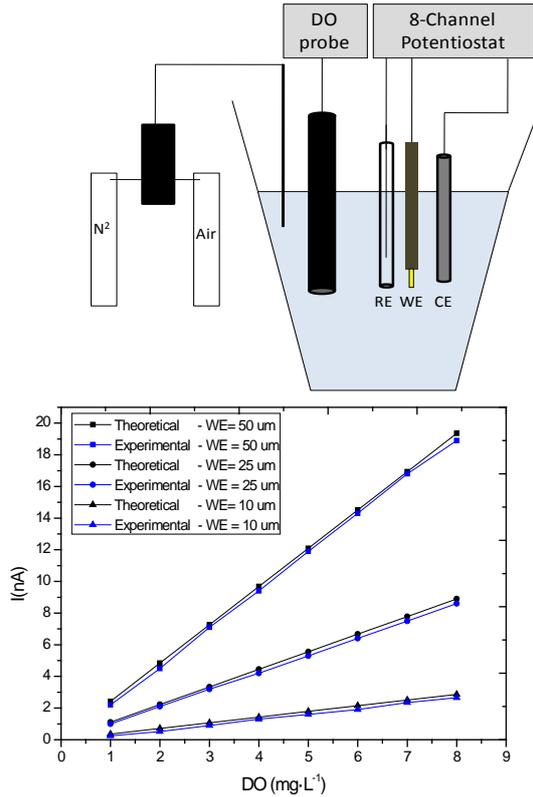


Fig 2. (a) Calibration set-up; (b) Theoretical and experimental calibration curves obtained for the reduction of oxygen for the three different designs.

The DO concentrations can be estimated experimentally from the microelectrode calibration response, or theoretically from the equation for recessed microelectrodes that Bond et al. [9] suggested:

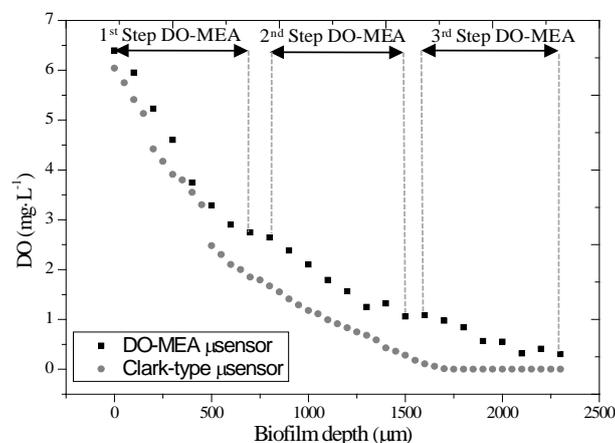
$$I_{recessed} = \frac{4nFDcr}{\frac{4L}{\pi r} + 1} \quad (1)$$

Where  $n$  is the number of electrons transferred during the oxygen reduction reaction,  $F$  [ $\text{C} \cdot \text{mol}^{-1}$ ] is the Faraday constant,  $D$  [ $\text{m}^2 \cdot \text{s}^{-1}$ ] is the DO diffusion coefficient,  $r$  [m] is the microelectrode radius,  $c$  [ $\text{mol} \cdot \text{m}^{-3}$ ] is the DO concentration and  $L$  [m] is the height of the passivation layer producing the recess. The experimental results were in good agreement with current values obtained from eq. (1) as shown in Fig 2b, considering a passivation recess height of  $1.9 \mu\text{m}$ . The experimental obtained sensitivities were: for the electrode diameter of  $50 \mu\text{m}$ ,  $2.41 \pm 0.08 \text{ nA} \cdot \text{mg DO}^{-1} \cdot \text{L}$  ( $n=20$ ); for the electrode diameter of  $25 \mu\text{m}$ ,  $1.10 \pm 0.08 \text{ nA} \cdot \text{mg DO}^{-1} \cdot \text{L}$  ( $n=5$ ) and for the smallest diameter of  $10 \mu\text{m}$ ,  $0.35 \pm 0.02 \text{ nA} \cdot \text{mg DO}^{-1} \cdot \text{L}$  ( $n=5$ ). The sensors display excellent linearity between  $0\text{-}8 \text{ mg DO} \cdot \text{L}^{-1}$  range with correlation factors ( $r^2$ ) greater than 0.99 for all three microelectrodes sizes. This analysis was completed studying the repeatability of the measurement resulting in a good behavior.

### 3. Biofilm DO profile

The suitability of the developed DO-MEA microsensors for biofilm monitoring was evaluated experimentally in a heterotrophic aerobic biofilm grown in a FPB. DO profiles were obtained using DO-MEA microsensors and compared to the commercial Clark-type microsensor, widely applied and validated for biofilm monitoring [10]. The thickness of heterotrophic biofilms, typically around 1 mm, allowed using any of the three different DO-MEA designs. The bigger diameter WE was chosen for this experimental. Fig. 3 shows the DO profiles that were obtained using DO-MEA compared to the commercial Clark-type microsensor. DO-MEA exhibits a good response with the same trend as Clark-type but with the advantage of the significant reduction of the acquisition time of the complete profile. With DO-MEA, 8-points can be obtained simultaneously in a single measurement in 15 seconds and only

3 steps were necessary to cover the total depth of the biofilm. For the Clark-type microsensor, each measurement was made every 50 $\mu\text{m}$  using a 3D micromanipulator. Both sensors were tested maintaining the same position within the biofilm.



**Fig. 3** Microprofiles of DO within an aerobic heterotrophic biofilm using the DO-MEA microsensor (■) and the commercial Clark-type r (●).

### 3. Conclusions

The technological improvement and the versatility of the standard microfabrication techniques open the possibility to multiple studies of biofilms with different thickness, even in micro-scaled biofilm reactors, at a low cost per sensor and high robustness. The novel DO-MEA sensor showed a good performance providing reliable instantaneous information of the activity inside biofilms. The developed DO-MEA sensor overcomes most of the commercial Clark-type microsensors drawbacks, and exhibits a good response with the same trend as Clark-type but with the advantage of the significant reduction of the acquisition time, enabling simultaneous measuring. This novel sensor represents an essential tool to record a biofilm profile in a single measurement. For opening DO-MEA applicability, the sensor fabrication in a thinner substrate and the protection of the electrodes with an oxygen-permeable membrane are ongoing.

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