Development of a novel microsensor for the study of oxygen profiles in biofilms

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ABSTRACT

Understanding of the processes taking place inside biofilms is a key parameter to progress in the optimization of biofiltration technologies. This study was conducted with the aim of developing a novel dissolved oxygen (DO) microsensor specially designed for biofilms monitoring. The microsensor was fabricated through standard photolithography techniques, resulting in a microelectrodes array (MEA) of 11 gold circular working electrodes, with a diameter of 50 µm, and a gold reference electrode, which allows obtaining a snapshot oxygen profile of 1 mm of depth. The performance of the sensor was fully characterized under different conditions, in which the sensor presented high sensitivity and repeatability, and low detection and quantification limits. Monitoring of sensor performance showed a stable and reliable response. The developed sensor was used in obtaining microprofiles in an aerobic heterotrophic biofilm, showing similar response to Clark-type commercial microsensors. These studies concluded that the novel MEA sensor for DO monitoring allows obtaining oxygen profiles within biofilms, becoming a useful tool for the research of many biological applications.

KEYWORDS

Microelectrodes array (MEA), dissolved oxygen, biofilm profiling, potentiometry

INTRODUCTION

Optimization and control of biofiltration processes, both in biofilters and biotrickling filters, can be performed by different mechanisms. In this sense modelling has become a useful tool for the study and selection of the optimal operating conditions. Hitherto models used in the description of the degradation processes in BF and BTF have been usually validated from measurements in the liquid and gas phases (Kim and Deshusses, 2003), due to the unavailability of performing measurements inside biofilms, where pollutants degradation reactions take place. The knowledge of the biological processes can be enhanced by the measurement of different chemical species within these biofilms. Oxygen is one of the most important species to monitor, since it is the primary electron acceptor in biological reactions (aerobic conditions). By studying its concentration inside the biofilms, characteristic profiles are observed, which can show the

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existence of different zones (Okabe et al, 1999), which become extremely important when understanding the biodegradation processes. In addition, it is possible to calculate parameters describing mass transfer [Ning et al., 2012] and biokinetics phenomena [Zhou et al., 2012] that allow developing more rigorous models than existing ones.

When performing biofilm characterization studies, microsensors are useful specific tools for the determination of the chemical parameters with a high spatial resolution. The most common oxygen microsensors are Clark-type [Revsbech and Jorgensen, 1986]. These sensors, based on Clark type macroelectrode [Clark et al., 1953], consist of an electrochemical system, comprising a reference electrode, a counter electrode and a gold working electrode, in a KCl solution. This system is isolated from the medium by a selective membrane. Oxygen molecules diffuse through the membrane, formed from gas permeable material such as silicone, to the cathode where they are reduced. The current generated on the cathode surface is measured and related to oxygen concentration. A negative potential must be applied to the cathode to provide electrons necessary in the electrochemical reactions. This potential, corresponding to the global reaction, is around -0.80V (vs Ag/AgCl).

Clark-type microsensors are handmade from pulled glass Pasteur pipettes, giving sensors with tips smaller than 10 microns. The manufacturing process causes limitations in the application of the sensor [Ching-Chou et al. 2005], being the most important the low repeatability of the sensors, the high fragility and the high cost per sensor.

The aim of the work presented herein is to develop a new oxygen microsensor based on MEMS (Microelectromechanical Systems) technology, overcoming the limitations presented by Clark-type microsensors. Microtechnologies involve the possibility of shrinking the dimensions to the scale of microelectronic circuits, and thus, it opens new industrial applications in numerous fields. They are rapidly becoming a key element in the development of advanced instrumentation for analytical research. The sensors manufactured by employing the microfabrication technology exhibit many advantages: mass-production of sensors with good-reproducibility, which at the same time decreases the production costs of these devices; and integration in a compact size with higher performance. In the present work, the design used for the sensor manufacturing is an array of multielectrodes (MEA) that allows obtaining a microprofile of dissolved oxygen (DO) in a single measurement, without the need of a micro-positioning system. Microsensors developed were used in the characterization of a heterotrophic biofilm cultivated in a laboratory-scale reactor.

EXPERIMENTAL METHODS

Microfabrication of DO MEA sensor

The oxygen microsensor designed and fabricated for the present study is shown in figure 1. It consists of an array of 11 gold circular microelectrodes, with a diameter of 50µm, separated from each other by 50µm, and a gold macroelectrode, in parallel to the array, with a surface of 0.115 mm^2. The eleven small microelectrodes are designed as working electrodes, while the rectangular one is designed to work as pseudo-reference. Sensor configuration has been specially designed in order to obtain a snapshot profile.

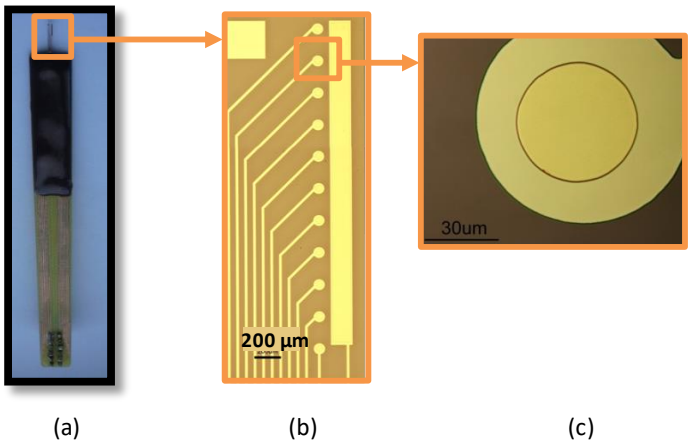

Figure 1. Description of the oxygen sensor designed and manufactured for biofilms monitoring: (a) Picture of the sensor (PCB and tip), (b) picture of the tip showing the array of working electrodes (circular electrodes), and the pseudo-reference electrode (rectangular electrode and (c) picture of one electrode.

The microsensors have been fabricated in the Clean Room facilities at the National Centre of Microelectronics in Barcelona (Spain), through standard photolithography techniques (Gabriel et al., 2007).

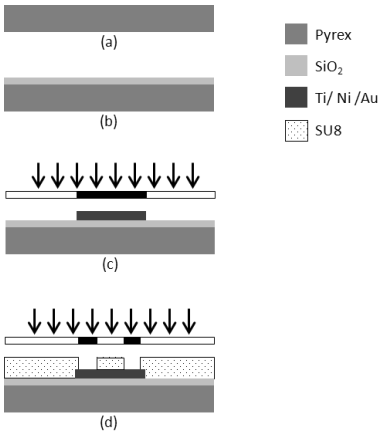

Figure 2 Schematic of the main steps of the DO array electrodes fabrication: (a) initial Pyrex substrate; (b) deposition of a SiO_2 layer; (c) patterning of the Ni/Ti/Au electrodes, strips and connection pads; (d) passivation of the electrodes by a SU8 layer except for the electrodes and the connection pads;

The electrodes are fabricated similarly as shown in Figure 2. This figure describes the electrode fabrication from 2a to 2d. The starting point is a 4 inch Pyrex wafer of 500 µm thickness (Fig. 2a). First, a 1.5 µm thick SiO_2 layer is deposited by plasma-enhanced chemical vapour deposition (PECVD) (Fig. 2b). Then, electrodes, contact pads and strips connection pads are patterned after a photolitography, a Ni/Ti/Au deposition (15/15/150 nm) and a lift-off process (Fig. 2c). In order to insulate the metal tracks, a SU-8 layer is processed on top of the wafer (Fig. 2d). This 1 µm thick passivation layer also defines the area of the microelectrodes which was designed to be 50 µm in diameter. Once the wafer is fabricated, sensors are encapsulated before the electrochemical characterisation of electrodes. First, the wafer is diced by a dicing saw. Then, they are glued to a previously fabricated Printed Circuit Board (PCB). Connection pads are wire-bonded and wires are protected with an epoxy based resin, as shown in Figure 1.

The electrodes array manufacturing was performed using a batch process. Approximately 440 microelectrodes arrays can be obtained from each pyrex wafer. Therefore, this manufacturing process simplifies the sensor fabrication, increasing uniformity and repeatability of the sensor and saving time and costs.

The resulting microsensor is 1050 microns of length, with the 11 electrodes for oxygen detection. The array configuration allowed obtaining an oxygen profile of a millimetre of length in a single measurement.

DO microelectrodes characterization

Calibration and characterization of the sensor were performed without using a Faraday cage, because despite the magnitude of the measured currents, the system provides a response without electromagnetic interference. The set up used in these stages is schematically shown in figure 3.

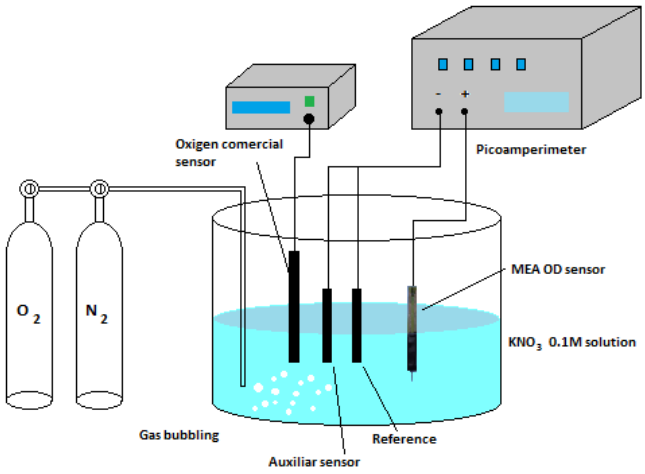

Figure 3.Schematic diagram of the calibration set-up.

Electrodes were polarized, in a first stage, with a commercial external Ag/AgCl reference electrode (6.0726.107 Ag/AgCl (KCl), Metrohm), and with an external auxiliary platinum electrode (6.0351.100 Pt-ring electrode, Metrohm), or with the internal pseudo-reference in a second stage. The polarization voltage source and the current input were provided from a multi-channel Electrochemical Analyzer (1030A, CH-Instruments), connected to an 8-channel multiplexor, that allowed only working simultaneously with eight electrodes. The polarization voltage applied on the working microelectrodes (cathodes) was studied separately for each reference system. Negative potential must reduce totally the oxygen dissolved in the measurement medium (equation 3), on the electrodes surface. Optimal value of the potential was determined according to sensor sensitivity analysis.

$$O_2 + 2e^- + H_2O \rightarrow HO_2^- + OH^- \quad [1]$$

$$HO_2^- + 2e^- + H_2O \rightarrow 3OH^- \quad [2]$$

$$O_2 + 4e^- + H_2O \rightarrow 4OH^- \quad [3]$$

Calibration of the sensor was performed comparing the current from the oxygen reduction on the cathodes surfaces, with the oxygen concentration measured with a commercial probe (Oxi 325, WTW). Dissolved oxygen was adjusted aerating pure air (21% O_2) and pure nitrogen (0% O_2) in a saline solution (measurement medium). Characterization of the sensor consisted on the study of its calibration in different conditions, in order to determine their applicability ranges.

Clark-type microsensor

Commercial Clark-type oxygen microsensors used in the study were purchased at Unisense (Denmark). The OX25 sensor used in this work has a tip diameter of approximately 25 µm. Electrodes were connected to a 4-channel amplifier (MicrosensorMultimeter, Unisense, Denmark) and polarized at -0.80V (vs Ag/AgCl). Data acquisition was performed using data acquisition software (Sensor Trace Basic, Unisense, Denmark) which resulted in real-time display of oxygen profiles in a computer. Linear two-point calibrations in the measurement medium solution were performed. Oxygen saturation conditions, taking into account salinity and temperature, were achieved aerating solutions with standard air (21% O_2). In addition, anaerobic conditions were obtained by adding Na_2SO_3 to the solution.

Sensor positioning within the biofilm was possible through the use of a three-dimensional micromanipulator (MM33-2, Unisense, Denmark), with a precision in z-axis of 10 µm, and in x-/y-axis of 100 µm.

Development of an aerobic heterotrophic biofilm

Response of the novel sensors and Clark-type ones was compared in the study of an aerobic heterotrophic biofilm, in order to check the robustness and reliability of MEMS-based microelectrodes. The biofilm used in these studies was grown in a Flat Plate Reactor (FPR), manufactured in methacrylate in the National Center of Microelectronics (Barcelona, Spain). The reactor was inoculated with active sludge from a pilot reactor treating wastewater. The biofilm growth was enhanced by feeding glucose, a substrate rapidly assimilated by biomass. The reactor, which permits

growing biofilms under well-defined conditions and using microsensors to quantifying chemical gradients in biofilms, was designed according to Beyenal and Lewandowski (2007). The reactor consisted of an open channel of 20 cm of length and 3.5 cm of width, partially filled by 5 mm of water. The reactor was operated reproducing the operating conditions of an industrial biotrickling filter. Mineral medium [Dorado et al., 2012] was fed to the reactor by a peristaltic pump (MCP Standard, Ismatec) with a flow rate corresponding to a liquid phase residence time from 4 to 8 hours. The liquid phase linear velocity was maintained between 0.05 and 0.2 $m \cdot h^{-1}$, regulating the recirculation flow rate driven by a peristaltic pump (Miniplus 3, Gilson).

RESULTS

Study of the DO MEA sensor response

Dissolved oxygen sensor performance and response was characterized in a validation stage, previous to sensor application in biofilm studies. The initial results showed that an initial cleanup of the electrodes was necessary due to the exposure of the sensor to the medium, which causes dirt deposition on the electrodes reducing its sensitive area [Kang and Rowntree, 2007]. Sensor activation was optimized by immersing the sensor in a solution of hydrogen peroxide (25% of volume) and 0.1 M potassium hydroxide (75% of volume) for one hour [Fischer et al., 2009]. It was necessary to repeat this procedure three times to obtain an optimal response of the sensor.

The polarization study revealed that the highest sensor sensitivity was obtained applying a potential of -0.85V on the working electrodes using the external reference system, and a potential of -0.40V when using the internal pseudo-reference. Based on these results it was possible to calibrate the sensor. The calibration curves for the tested microelectrodes are shown in figure 4.

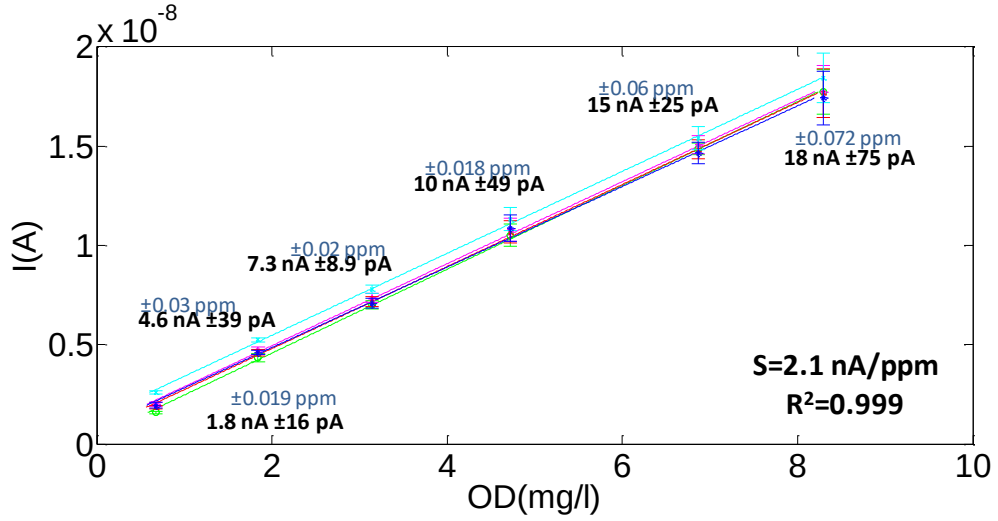

Figure 4.DO MEA Microelectrodes calibration. Results correspond to the average of ten calibrations for each electrode. Deviations indicated in blue on the chart refer to the measurement repeatability, indicated in black the electrodes response repeatability. The parameter S corresponds to the measured sensor sensibility, and the parameter R^2 corresponds to the Pearson coefficient.

Sensor calibration was performed in a 0.1M KNO_3 solution, using 6 different oxygen concentrations, for eight of its electrodes, therefore obtaining eight linear regression equations in a sensor calibration. This procedure was repeated ten times in order to statistically analyse the sensor response.

Results showed a high correlation coefficient for all electrodes, in all cases, in the DO range of 0 to 8 $mg \cdot L^{-1}$. The sensor sensitivity was calculated as the global sensitivity of eight electrodes, obtaining a value of 2.1 $nA \cdot mg^{-1} \cdot L$ higher than other similar sensors [Lee et al., 2007a]. The time for 90% response found was 15 s, which is shorter than similar microelectrodes [Lee et al, 2007b], marking an important improvement. From the statistical study it is possible to observe a high repeatability of the sensor response, with deviations smaller than 0.072 $mg \cdot L^{-1}$. This study was completed by determining the limits of detection and quantification calculated as 0.15 $mg \cdot L^{-1}$ and 0.25 $mg \cdot L^{-1}$ respectively.

The sensor stability was studied in the second characterization stage by analyzing the sensor response along time. This monitoring of the sensor was performed using two different methods. The first method consisted of monitoring the

sensitivity of the sensor after an initial activation, and the second one consisted of monitoring the sensitivity cleaning the sensor before each analysis. From this study it was observed that the sensor maintained 90% of its initial sensitivity 600 hours after its activation. Additionally it was determined that the sensor could be reactivated more than 8 times without modifying the sensor response, which corresponds to an operating lifetime of at least half a year.

Sensor characterization was finished by determining the effect of the mineral medium composition in the sensor response. Results indicated that the ionic species nature in the growth medium solution have no effect on the response of the sensor. However, it was possible to observe a close relationship between salt concentration and sensor sensitivity, which decreases by 15% when the medium was diluted ten times. Therefore a minimum ionic strength is required for optimal sensors performance. Finally, the pH effect was studied, finding that the sensitivity increases when the pH is alkaline. The microsensors show deviations on their normal response at pH below 3. This is due to secondary electrochemical reactions that take place in these conditions. Then, the recommended pH of operation ranges between 3 and 12.

Application of MEA sensor to microprofiles measurements in biofilms

The viability of the oxygen MEA sensor developed in this study was evaluated. DO microprofiles were obtained and compared using both MEA sensors and commercial Clark-type microsensor under the same conditions. An aerobic heterotrophic biofilm was grown in the FPR and both microsensors were tested after a suitable biofilm thickness was observed. Identical positioning of the sensors in the biofilm was achieved using a 3-D micromanipulator. DO microprofiles were measured at 100 µm intervals for MEA sensor, in a single measurement, and at 50 µm intervals for Clark-type microsensor, using the micromanipulator.

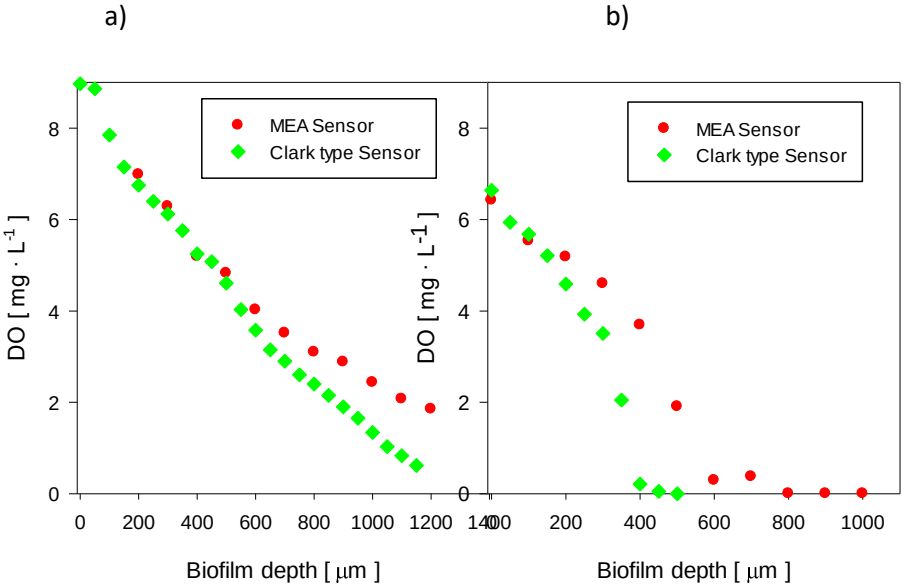

Figure 5. DO microprofiles. (a) Microprofiles measured under endogenous conditions. (b) Microprofiles measured during substrate consumption.

Figure 5 shows the microprofiles obtained using both MEA and Clark-type sensors. From the study of these profiles it is possible to determine the effective depth of the biofilm. Under endogenous conditions (figure 5a), oxygen decreases slowly from the biofilm-liquid interface up to the anaerobic limit, resulting in a biofilm effective depth of approximately 1200 µm. The effect of substrate feeding to the reactor can be observed in figure 5b by an increase in the oxygen consumption within the biofilm at a depth of 600 µm, consequently reducing by roughly 50% the effective depth of the biofilm.

Regarding to the differences in MEA and Clark-type microsensors profiles, these were most probably caused by MEA sensor width which resulted in disturbances in biofilm structure, allowing the entry of medium between the biofilm and the electrodes, and by slight differences of positioning of the electrodes. However, technologically the sensor width is a problem easy to overcome because exist several alternatives (thinner silicon or pyrex wafers, or polymeric substrates) easy to implement that would help to decrease the damage caused by the penetration of the MEA. Although both sensors response exhibit the same trend, thus confirming the suitability of the developed MEMS-based microelectrode further testing is warranted to completely validate the sensor developed.

CONCLUSIONS

A novel oxygen microsensor has been designed and manufactured using MEMS technology. This technology enables adapting the sensor design to the applications needs, selecting electrodes diameter and the separation between them. The sensor, specially designed for biofilms monitoring, allows obtaining profiles on 1 mm length in a single measurement. Calibration tests revealed a high sensitivity and repeatability and low detection and quantification limits. Sensor monitoring showed that the sensor presents a stable and reliable response. The sensors were tested by measuring DO microprofiles within an aerobic heterotrophic biofilm, achieving good results, comparable to those obtained with commercial microsensors.

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