Micro-needle implantable electrochemical oxygen sensor: *ex-vivo* and *in-vivo* studies

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Abstract

Oxygen is vital for energy metabolism in mammals and the variability of the concentration is considered a clinical alert for a wide range of metabolic malfunctions in medicine. In this article, we describe the development and application of a micro-needle implantable platinum-based electrochemical sensor for measuring partial pressure of oxygen in intramuscular tissue (*in-vivo)* and vascular blood *(ex-vivo)*. The Pt-Nafion® sensor was characterized morphological and electrochemically showing a higher sensitivity of -2.496 nA/mmHg (-1.495 nA/µM) when comparing with its bare counterpart. Our sensor was able to discriminate states with different oxygen partial pressures *(pO_2)* for *ex-vivo* (blood) following the same trend of the commercial gas analyzer used as standard. For *in-vivo* (intramuscular) experiments, since there is not a gold standard for measuring pO_2 in tissue, it was not possible to correlate the obtained currents with the pO_2 in tissue. However, our sensor was able to detect clear statistical differences of O_2 between hyperoxia and hypoxia states in tissue.

1. Introduction

Hypoxia is the term used to describe lower than normal oxygenation levels in tissues, which are symptoms of a number of pathologic conditions that affect a localized tissue area, but if an overall deficit of O_2 is produced, the whole body is critically affected (Reinhart and Eyrich, 1989). *In-situ* monitoring of O_2 levels in tissue is crucial for medical proactivity and decision making for addressing severe complications that an irregular O_2 supply can cause. However, currently there is not a gold standard device or technique for measuring O_2 in tissue.

In clinical practice, arterial blood gas (ABG) analyzers are the gold standard for measuring dissolved oxygen and carbon dioxide. They are based on electrochemical sensors and represent an essential tool for checking the oxygenation status and acid-base balance. Although these devices are commonly used, they are limited to blood analysis, and so limited to many applications (van der Weerd et al., 2017).

Electrochemical sensors have been used in clinical analysis for decades due to their high sensitivity and selectivity, as well as their miniaturization capabilities and low cost. Among the electrochemical sensors, the Clark-type oxygen sensor is the most used for dissolved O_2 detection in liquid sample or gas. As O_2 is an electroactive molecule, its cathodic reduction produces a current that is proportional to the O_2 concentration.(Clark, 1956; Nei, 2007; Severinghaus and Astrup, 1986) Different microfabrication techniques and their integration with electrochemical systems have been developed for monitoring O_2 in biological systems. For instance, a miniature Clark type-sensor was integrated within a microstructure of poly(dimethylsiloxane) (PDMS) for measurement of the adhering cells´ respiration. Although the microstructure formed by a PDMS chamber and a O_2 permeable membrane showed its instability over a long period of time, this sensor exhibited a fast response, about 6.8 sec, to changes in O_2 concentration, which was convenient to monitor the kinetics of O_2 variation in biological samples.(Wu et al., 2005) In another report, Lee et al. developed a needle-type gold microelectrode array sensor of dissolved O_2 in bacterial films. The microfabricated system exhibited a fast linear response, about 15 sec, in the 0–21% O_2 range, and allowed *in-situ* measurements by penetrating biological or environmental samples, such as biofilms.(Lee et al., 2007) Recently, an implantable platinum-based O_2 sensor was developed and validated in *in vivo* conditions for monitoring peripheral tissue O_2 in rats and pigs as animal models. The system showed good linearity and sensitivity (Finnerty and Bolger, 2018). Noble metals, such platinum and gold, are the most common transducer platforms for O_2 sensing; however carbon-based materials have also been used for this purpose and applied for real-time O_2 monitoring in brain tissue.(Bolger et al., 2011a, 2011b).

In this article, the development of a wireless and implantable micro-needle O_2 electrochemical Clark-type sensor designed for easy clinical manipulation is presented (Figure 1). The micro-sensor comprising of three electrodes, each of 150 µm in diameter, encapsulated in a biocompatible tubing (final diameter ~500 µm), for ease insertion through a needle and then to be implanted into any part of the body. Once the O_2 sensor is inserted and used for the analysis, it can be painlessly extracted. The developed platform was tested by surgical incision into adult rabbits for intramuscular tissue measurements and blood *ex-vivo* sampling by modulating the fraction of inspired O_2 by the animal.

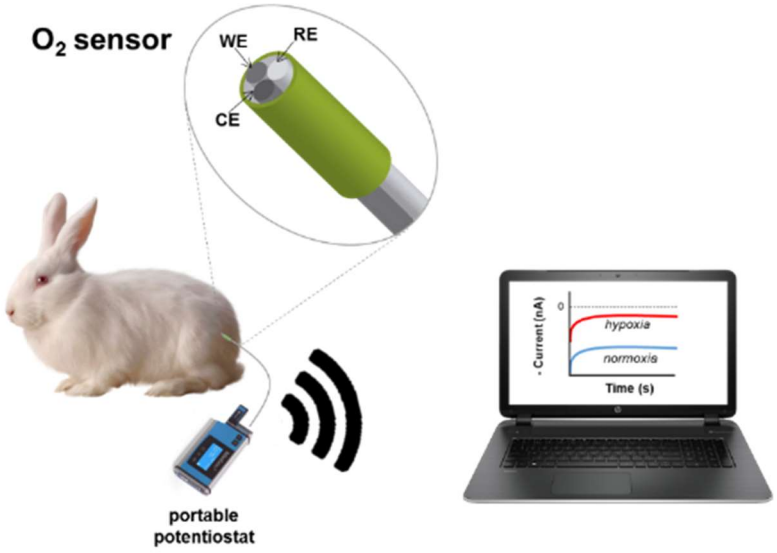

Figure 1. Scheme of the developed wireless implantable electrochemical oxygen sensor tested in rabbit model

2. Methods and materials

2.1 Materials

Potassium chloride (KCl), Nafion® perfluorinated resin solution 5 wt. % and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Spain). Platinum and silver polytetrafluoroethylene (PTFE) insulated wires, used for the electrodes, both at 99.9% purity and with 0.125 mm in diameter (~0.150 mm diameter with coating), were purchased from Advent Research Materials (UK).

Electrochemical measurements were performed with a custom-built multipotentiostat from PalmSens (Germany). The dissolved oxygen concentrations *in-vitro* were measured using a

PreSens oxygen meter (model Oxy-4 ST prototype, Germany). Surface electrodes were observed by using an Ultra-High-Resolution Field Emission Scanning Electron Microscope (model Nova NanoSEM, FEI, USA). The *ex-vivo* measurements in blood were analyzed as standard with EPOC® reader and EPOC® BEGM test card (Alere/Siemens Healthcare, Barcelona).

2.2 Oxygen sensor preparation

The oxygen sensor consisted in three electrodes: a bare Pt wire as counter, a fabricated (Ag/AgCl) wire as reference, and a membrane-modified Pt wire as a working electrode. The insulating layer of one end of metallic wires (Pt or Ag) was carefully removed to enable electrical connections to the potentiostat. The other end of the wires were modified as follows: the Pt wire was prepared as the working electrode with two layers of Nafion®, as received, was deposited on the metallic surface and dried overnight in an argon chamber. Then, it was cured at 100 ºC for 1 h. For preparing the reference electrodes, three Ag wires were interconnected at their ends and anodized in a saturated solution of 3 M KCl for 2 min with a 9 V battery, and then dried overnight. The reference electrode was protected with a Nafion® membrane by immersion of the modified Ag wire in 5 % w/w Nafion® solution for 2 minutes. Finally, it was cured at 100 ºC for 1 h.

The three electrodes were assembled within a polyether ether ketone (PEEK) tubing with an external diameter of 500 µm and kept in argon chamber for further use. Before use, Nafion®-modified sensors were left to swell in PBS buffer for 20 min.

2.3 Electrochemical O_2 monitoring

2.3.1. In-vitro measurements

Cyclic Voltammetry (CV) was performed from +0.2 to -1.0 V at 100 mV/s. Chronoamperometry (CA) was performed at a fixed potential of -0.7 V for 300 sec. For all the experiments, the measurements were taken at least by triplicate ($n \geq 3$) using different sensors.

All *in-vitro* calibrations were performed with PBS buffer with different oxygen concentration by purging with N_2 or O_2 for at least 30 min. The calibration was performed with three different oxygen concentrations, unless otherwise noted. *In-vitro* blood analysis was performed with extracted blood from rabbit. For low O_2 concentrations in blood, the sample was purged with N_2 and vigorously stirred to avoid the foam. N_2 displaces the O_2 in the samples but not completely, being 10 % the final concentration that was characterized with the optical sensor PreSens (PreSens, n.d.).

2.3.2. In-vivo measurements

A total of six New Zealand white male rabbits (2.5-3 kg body weight) were included in this study. Animals were provided by a certified breeder (Granja San Bernardo, Navarra, Spain) and acclimated for five days before surgery. For this study, the measurements were taken at least by triplicate ($n \geq 3$) in each stage (*i.e.* hyperoxia, hypoxia, recovery) and using one sensor per animal.

Animals were individually caged (dimensions 70x75x40 cm) with visual, auditory and olfactory contact allowed among different individuals. Housing was performed under conventional conditions in an environmentally controlled room. Animals had free access to tap water, standard commercial pelleted diet (2030 Teklad Global diet, Envigo) and hay.

Animal handling and all experimental procedures were performed in accordance with applicable regulation and guidelines and with the approval of the Animal Experimental Ethics Committee of the University of Barcelona (ref. 236/16) and the competent authority Generalitat de Catalunya (ref. 9349).

Animals were subjected to general anesthesia and premedicated with buprenorphine (0.03mg/kg, SC), ketamine and xylazine (35mg/kg and 5mg/kg respectively, SC). Induction was performed by IV administration of propofol (5mg/kg). A face mask was used for oxygen (100% oxygen, 2L/min) and isoflurane (2%) administration. Trachea and carotid artery were exposed through a midline incision in the ventral cervical area. A 3-4mm tracheostomy was performed and a 3.0-endotracheal tube was inserted. Carotid artery was carefully dissected and cannulated with a 22G-IV for serial blood sampling (see supporting information, figure S4). Mechanical ventilation was set at 20 breaths per minute, 1:2 of inspiration/expiration ratio and 2L/min-inhalatory flux (100% oxygen, 2% isoflurane). Body core temperature was maintained with an electric pad. Heart rate, respiratory rate, oxygen saturation, body core temperature and reflexes were monitored and recorded during the whole experiment.

For the sensor insertion, a 2-3 cm incision was performed in the skin and fascia muscularis in the right femoral quadriceps muscle. After exposing the muscle, one or two sensors per rabbit were implanted through the incision and secured to the parenquima using simple knots (silk, 3/0). Skin was closed using a running suture (silk 3/0).

Thereafter, animals were subjected to acute hypoxia by reducing the fraction of inspired oxygen (FiO_2). The protocol consisted on a period of hyperoxia (20 min), hypoxia (60 min) and recovery (40 min). Upon animal stabilization, the protocol started with 20 min of hyperoxia, or basal period, breathing 100% oxygen (mechanical ventilation configured as mentioned above). During hypoxia, animals breathed a mix of 10% Oxygen and 90% Nitrogen (10 rpm, 1:2 I:E and 1L/min inhalatory flux) for 60 min. In the recovery period, animals breathed 100% oxygen as in the basal period. An IV injection of rocuronium bromide (0.6 mg/kg, IV) was administered when needed.

Amperometry measurements were stabilized prior to the hypoxia induction to set a current baseline. Once the current was stabilized, several measurements of 300 s were carried out during each state ($n \geq 3$) whenever possible.

Considering the medical application of this sensor, and to avoid cross-contamination between patients, the sensor was designed for single use at low cost and adaptable to the portable reader. So, the sensors were not reused for *in-vivo* testing and a new surface was required with each-subsequent calibration every time.

2.3.3. Ex-vivo measurements

Ex-vivo experiments were performed using artery blood samples (0.2 mL) from the carotid artery, which were extracted at 10-15 min intervals during the *in-vivo* hypoxia protocol. For this study, the measurements were taken at least by triplicate ($n \geq 3$) in each stage (i.e. hyperoxia, hypoxia, recovery) and using one sensor per animal. Samples were immediately processed in parallel using the developed sensor and the EPOC® blood analysis device.

2.5 Data treatment

PS Trace 4.8 software was used for the electrochemistry and data collection. Chronoamperometric data was normalized to reduce variability between sensors. Oxygen partial pressure were reported primarily in mmHg to compare with EPOC device's units, used as our standard. Oxygen unit conversion was carried out with a tool provided by PreSens.(PreSens, n.d.)

One-way analysis of variance (ANOVA) was used to evaluate the statistical differences between multiple results. With a confidence value of $\alpha=0.05$, values $p \geq 0.05$ were considered statistically not significant (n.s.); those between $0.01<p<0.05$ were considered significant (*); $0.001<p<0.01$ were very significant (**); $0.0001<p<0.001$ were extremely significant (***) as well as those $p<0.0001$ (****). *t*-test was used to assess statistical differences between two sample means (two-tailed paired or unpaired, when appropriate).

3. Results and Discussion

3.1. Surface characterization

In this work, an implantable Clark-based O_2 micro-sensor was developed using a Pt wire, as transducer, and coated with Nafion®, which acts as a permeable membrane. The fabricated Pt-Nafion® oxygen sensor, as well as the Ag/AgCl reference electrode and the bare Pt counter electrode were characterized by scanning electron microscopy (SEM). All the metal wires used

as electrodes were insulated with biocompatible PTFE, to avoid biofouling effects when implanted into tissue. Figure 2A shows the magnified surface of the sensor at 330X, while Figure 2B-D, shows each electrode at 1300X, separately. Bare Pt wire as counter electrode (Figure 2B) shows a slightly striated metal surface produced by the transversal cut; the modified Nafion®-Pt electrode as working electrode shows a smooth surface covered with a thin layer of polymer (Figure 2C); and the Ag/AgCl reference electrode formed by anodically grown silver chloride crystals on the electrode, shows the rough surface with needle-type crystals growth on the silver surface covered with a layer of Nafion® (Figure 2D).

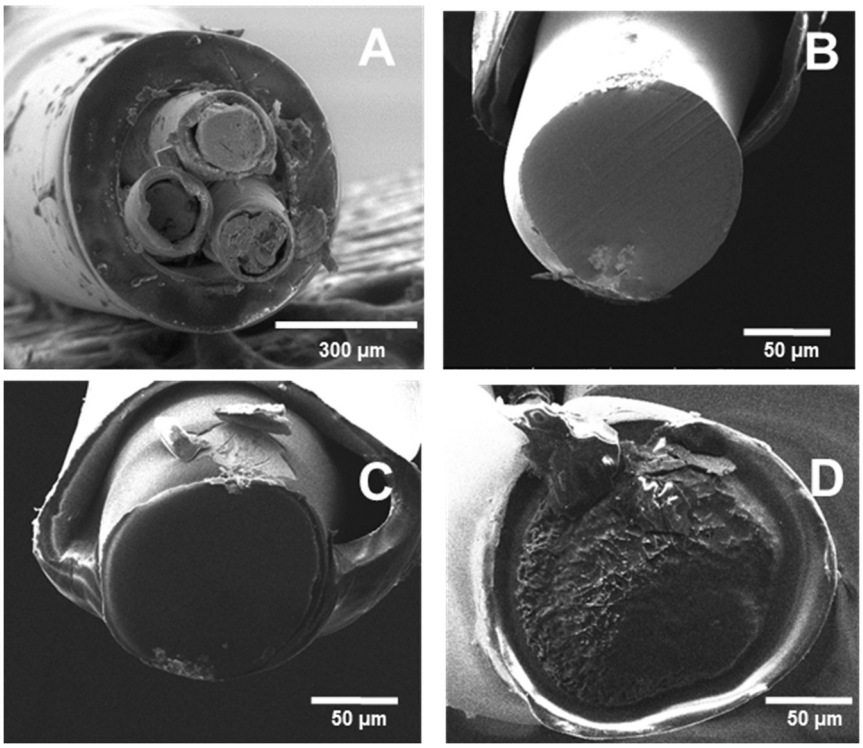

Figure 2. Scanning electron micrographs of: (A) Pt-Nafion® sensor; (B) bare Pt electrode; (C) Pt-Nafion® electrode; and (D) Ag/AgCl electrode.

3.2. Electrochemical characterization

The fabricated RE based on an anodized Ag wire coated with Nafion® was characterized by cyclic voltammetry with Pt WE and Pt CE in 50 mM of ferro-/ferricyanide solution, observing well defined reversible peaks around 0.13 V. Moreover, long-term stability studies of Nafion® coated RE and uncoated RE, stored in an ambient condition (room temperature and open-air), were performed over 18 months, to test the protective feature of Nafion®. The freshly prepared Nafion® coated RE gave a similar performance after 5 days and starts to present a potential shift after 18 months of storage. Meanwhile, the uncoated RE shown failure on the detection with

the decrease on reduction/oxidation faradaic current (See supporting information, Figure S2). These results show the importance on the Nafion® protection for the long-term stability of the Ag/AgCl RE, in addition to the biocompatible surface that offers as well as a non- interfering environment with the overall performance of the RE.

The developed implantable Clark-based O_2 micro-sensor based on Pt-Nafion® was characterized electrochemically. Nafion® is a biocompatible perfluorinated polysulfonate polymer that has been used in biosensors, especially as barrier for anionic interfering species. Additionally, Nafion® has the benefit of gas permeation through the polymer and reach the surface for subsequent reduction.(Heitner-Wirguin, 1996; Lawson, 1988; Zimmerman and Wightman, 1991) Depending on the material of the electrode, the reduction mechanism of oxygen may proceed either two- or four-electron processes.(Wu and Yang, 2013; Xiao et al., 2017) The latter is attributed to materials such as Pt, in which the oxygen can be reduced completely to water without the intermediate formation:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

In Figure 3, CV was conducted to evaluate the reduction potential of the oxygen at the bare and Pt-Nafion® sensor. As shown in Figure 3A, although the current increased as the O_2 partial pressure (pO_2) rose up, the bare Pt sensor did not exhibit a clear cathodic peak, while the Pt-Nafion® sensor showed a more defined O_2 reduction peak at approximately -700 mV; and the current increased proportionally to the pO_2 (Figure 3B). This fact may be due to the lateral hydroxide interactions on the bare Pt, which are avoided in the Pt-Nafion® electrodes due to the mutual repulsion between sulphonate groups, that make up this polymer, and anionic molecules.

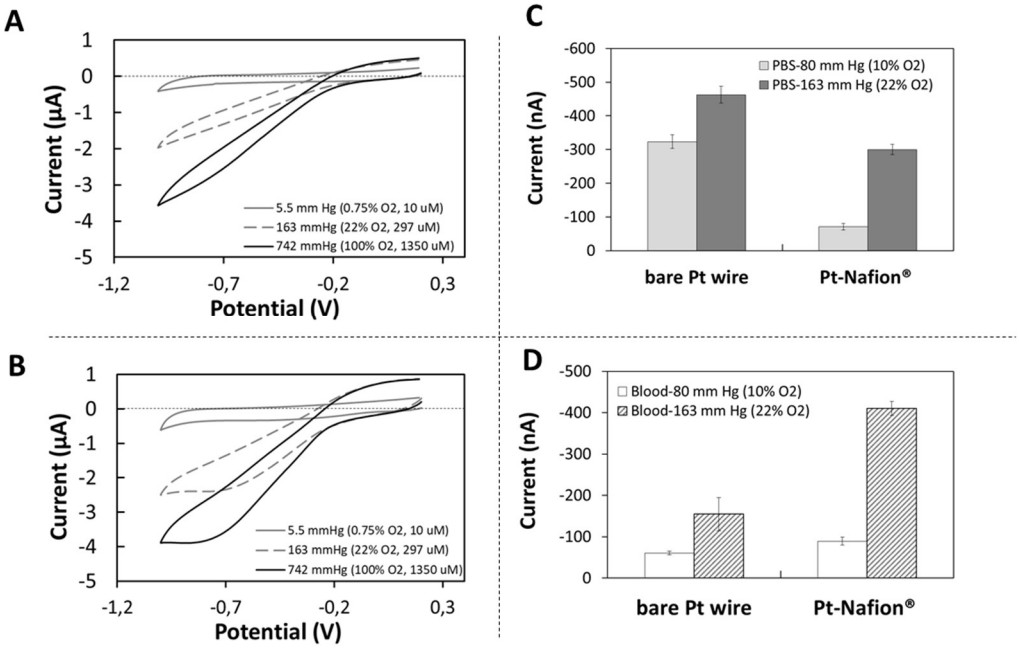

Figure 3. Cyclic voltammetry of (A) bare Pt and (B) Pt-Nafion® electrodes at different oxygen concentration: 5.5, 163 and 742 mmHg. Current bar graphic for bare Pt and Pt-Nafion® electrodes in (C) PBS and (D) blood oxygenated and artificially deoxygenated at 163 and 80 mmHg, respectively.

For determining the sensitivity of the O_2 sensors, calibration curves were in the range of 5.5 (0.75%) to 742 (100%) mmHg at 25ºC. O_2 calibrations in PBS buffer with bare Pt electrodes showed a sensitivity of (-1.673±0.144) nA/mmHg (-1.002 nA/µM, R^2=0.9713), while Pt-Nafion® sensor displayed a higher sensitivity of (-2.496±0.179) nA/mmHg (-1.495 nA/µM) with good linearity (R^2=0.996). Both sensitivities resulted significantly different (p=0.0003), demonstrating the better performance of Pt-Nafion® vs. bare Pt. When comparing Pt-Nafion® sensor results in PBS (Figure 3C), and with stored blood under different oxygen concentrations (Figure 3D), the benefits of Nafion® as a suitable membrane are clear. Because on one side, the Nafion® membrane does not affect the oxygen diffusion to the electrode surface, and on the other side, it blocks different elements in complex matrix, such as blood, with a benefit on the sensor sensibility. The Nafion® structure combining perfluoroalkyl and sulfonated groups enables selective blocking of the anionic species through electrostatic interaction with sulfonated groups, whereas the fluorocarbon hydrophobic moiety give a selectivity for the hydrophobic molecules (Bolger et al., 2011a). This point is also observed in our characterization of the O_2 sensor by cyclic voltammetry in a blood complex matrix, where no extra redox peaks are observed in the range of the voltage studied (see supporting information Figure S5). In addition to that, and even with more relevance for implantable systems, Nafion® offers biocompatibility and excellent anti-fouling, and anti-passivation properties. Calibration with rabbit's stored blood samples was carried out in a pO_2 range between 80 and 163 mmHg giving a sensitivity of (-3.8675±0.1282 nA/mmHg, R^2=0.996). As we expected, the bare Pt sensor was poisoned with the proteins and other components of blood reflecting a lower current difference at two distinct O_2 content values when comparing with the Pt-Nafion®, which exhibited a higher difference. In this work it was corroborated that an efficient Nafion® coating improved the sensitivity and selectivity in complex matrices. In table 1 is presented a comparison of our sensor with other Pt-based electrochemical sensors with different coatings tested in vivo. Similar sensitivities have been reported in these works, but Bolger et al. demonstrated the lower detection limits. However, the Bolger's platform has a very complex preparation process, being more time-consuming because requires an extra preparation of polymer solutions, more coating layers, and in some cases, further electropolymerization. On the other hand, Finnerty et al. developed

similar arrays that were used for in-vitro and in-vivo monitoring, but our setup has an easier handling for clinical use.

Moreover, differences can be observed with the Pt-Nafion® sensor´s sensitivity under different type of samples; buffer and blood. This fact may be due to the different contents and accessibility for O_2 reduction depending on the matrix. Chronoamperometry results shows different kinetics on the O_2 reduction related with the matrix used; being slower in the case of blood. Also, the reoxygenation after 300 seconds of the nitrogenated sample is null in the case of blood, meanwhile it is clearly observed in buffer (see supporting information, figure S3). The response to these behaviors may be found in the different natures of the matrix. Buffer contains O_2 from the atmosphere and in the case of blood, there are two O_2 sources; the atmosphere and hemoglobin, where the latter is more difficult to reach the entrapped O_2 for being reduced, decreasing the kinetics and its replacement from the atmosphere.

Table 1. Summary of comparisons of different Pt-based electrochemical sensors tested in vivo.

Sensor	***Linear range (µM)***	***Voltage (V)***	***Sensitivity (nA/µM)***	***Current density (nA/mm²µM)***	***Limit of detection (µM)***	***Reference***
Pt-Pt	0-1200	-0.80 (vs.Pt)	-0.64	50.4	0.30	(Finnerty and Bolger, 2018)
		-0.65 (vs.Pt)	-0.37	29.5	0.26	
ClinOx		-0.80 (vs. Pt)	-1.41	7.2	0.19	
Pt	0-1200	-0.65 (vs.SCE)	-1.12	91	0.08	(Bolger et al., 2011a)
Pt-PPD			-0.95	-	-	
Pt-Rhophlex			-1.02	-	-	
Pt-PMMA			-1.06	-	-	
Pt-Nafion®	9-1239 (5.5-742 mm Hg)	-0.70 (vs.Ag/AgCl)	-1.495 (-2.496 nA/mm Hg)	0.122 0.203 (nA/mm²mmHg)	4.57 (2,74 mmHg)	This work

As depicted in Figure 4, three cycles between 80 mm Hg and 163 mm Hg were carried out to evaluate the repeatability of the measurements pO_2. Response time is an important parameter to consider since the sensor needs to detect the changes of gas concentrations quickly. This parameter is limited by the time needed for the gas to diffuse through the membrane and reach

the electrode surface.(Xiong and Compton, 2014) Additionally, membrane-based sensors are strongly influenced by the type and thickness of the Nafion coating as reported by Clark (Clark et al., 1953). As shown in Figure 4, the current values for each pO_2 were similar and the time to reach at 90% (t_{90}) of the stabilized current was *ca*. 20 seconds. Although, the current changes from one pO_2 value to the other were immediate, the time to reach a stable current was longer.

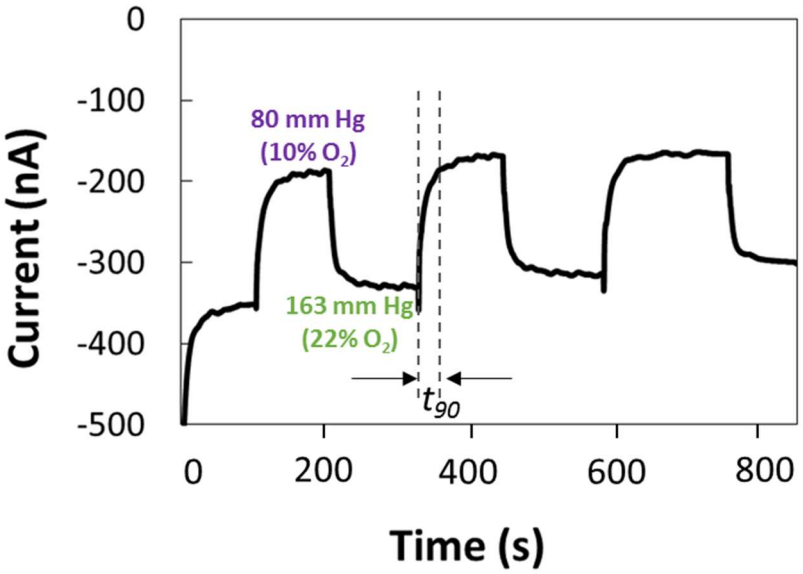

Figure 4. Chronoamperometry showing the reversibility of the current change at 80 mm Hg (10%) and 163 mmHg (22%)

3.2.1 pH, interferences and temperature effects

The physiological changes that occur under ischemia conditions comprise a shortage of blood supply on tissues and consequently a reduction of O2 provision to the tissue. The lack of oxygen to the cells produces anaerobic conditions that lead to the formation of lactic acid, with a reduction on the extracellular pH from 7.4 under physiological conditions to approximately 7.1-6.8. Moreover, under prolonged ischemia conditions oxidative stress is produced with the result of hydrogen peroxide outcome. The estimated physiological ranges of H2O2 in oxidative stress intracellular are between 1–10 nM up to approximately 100 nM. An estimated 100-fold concentration gradient from intracellular to extracellular is produced with certain changes depending on cell type (Sies, H., 2017). Thus, the extracellular peroxide concentration under oxidative stress range between 10 pM to 1nM, which is the concentration that may interfere our sensor in tissue. Since peroxide may affect the O2 redox (Mano, et al., 2018), cyclic voltammetry was performed with the Nafion-Pt sensor in the presence of 22% of O2 and 1-100

nM of peroxide to test the possible effect of peroxide in the O2 monitoring. As can be appreciated in Figure S6 just a small peak at -0.4 V appear due to the presence of H2O2 and negligible effects are observed in oxygen detection under these peroxide concentrations (figure S6).

In noble metals such as Pt, the O_2 reduction is carried out through a four-electron mechanism that involves proton transfer, which could be affected by the variation of pH during *in-vivo* experiments. Considering, the Pourbaix diagram and Nerst equation; E=59.1mV*pH, a theoretical shift of around 142 mV is expected (Pourbaix, 1966) For verifying the influence of pH in the performance of our sensor, CV was conducted in PBS at pH values of 4.0 and 7.4. Under the tested pH, the CVs recorded with the O_2 sensor shift 28 mV on the cathodic peak (see supporting information S1). The lower changes appreciated in our sensor may be due to the mitigation by the acidic Nafion, a proton donor that reduce the effects under low pH. Moreover, the acidic PTFE insulation used in the Pt wires increase even more this effect.

Nevertheless, potential shift duo to pH changes should not affect the O_2 sensors sensitivity, since the chronoamperometries are performed on the plateau of the cathodic wave. Results obtained for O_2 calibrations at pH 4.0 and pH 7.4, yielded sensitivities of (-2.350 ± 0.140) nA/mm Hg (-1.407 nA/ µM, R^2=0.997) and (-2.276±0.062) nA/mmHg (-1.362 nA/µM, R^2=0.973), respectively. No significant difference was observed for both calibrations (p=0.6230, paired t-test), indicating that the sensitivity of pO_2 sensing is not affected by pH. This agrees with a previous report on the independence of pH on the sensitivity of Pt sensors.(Bolger et al., 2011a)

It is known that membrane-based oxygen sensors are temperature-dependent, due to changes on the solubility of the gas and its diffusion coefficient produced by the effects of temperature (Bolger et al., 2011b). It has been reported that Pt electrodes showed a deviation of 3 % per 1ºC in brain tissue, but without impact on measurements because of the regulated cerebral temperature though (Bolger et al., 2011a). In order to determine if temperature could affect the sensitivity of our Pt-Nafion® sensors, calibrations were performed at 25 ºC and 38 ºC. Results showed that sensitivities at 38 ºC (-2.841±0.170 nA/mmHg, -2.044 nA/µM, R^2=1) were not significantly different (p=0.5097, paired t-test) from those at 25 ºC: (-2.893±0.139 nA/mmHg, -1.732 nA/µM, R^2=0.976) and were negligible.

3.2 Ex-vivo studies

The optimized micro-O_2-sensor in buffer was then used under real conditions with blood samples. Blood samples used for *ex-vivo* studies were extracted during the *in-vivo* experiments at the hyperoxic and hypoxic states, by modulating the fraction of inspired O_2 to modify the concentration of this gas in the animal blood. In this procedure, the initial value of oxygen was 100 % and was gradually reduced to hypoxia, which consisted in 10 % of oxygen and 90 % of nitrogen, passing through the different stages of the animal response. The recovery was achieved moving again to 100 % of oxygen. The reported results of *ex-vivo* and *in-vivo* are related with the animal response, considering blood measurements in parallel with a standard commercial EPOC® reader.

Metal electrodes are affected by poisoning when immersing in blood because of the adsorption of biological material over the surface, thus affecting the performance of the sensor.(Clark et al., 1953; Patrick et al., 2017) For this purpose, Nafion® membrane was used as a coating membrane on the working electrode of our O_2 micro-sensor, taking advantage of their O_2 diffusion capability, for filtering out unwanted particles because of its anti-biofouling properties, which is very important for *in-vivo* sensor implantation.

Figure 5A depicts the correlation between cathodic current and pO_2 measured with EPOC® in a range from 0 up to 600 mmHg, considering all the animals used for this study (n=6), as well as a sensor for each animal, resulting in a sensitivity of (-0.584 ± 0.091) nA/mm Hg (-0.420 nA/µM *,* R^2=0.476). In the graph, the data was collected in two main quadrants: "I" for hyperoxia- and "III" for hypoxia. Except for few data points, most of the hypoxia data points were within the "III" quadrant, that means currents below -30 nA and pO_2 values below 50 mmHg according to EPOC® device. A linear tendency is observed between 50 mmHg and 250 mmHg of pO_2, with a range of current from -30 nA to -100 nA. While hyperoxia and recovery values (see Figure 5A, quadrant I) showed a high dispersion due to the animal variability for bearing atypical pO_2. If hyperoxia values beyond 300 mmHg are not considered, the data then fitted properly in a linear fashion (Figure 5B). However, the number of animals and sensors decreased (n=2), showing a lower sensitivity of (-0.305$\pm$0.014) nA/mmHg (-0.219 nA/µM) and an acceptable linearity was exhibited (R^2=0.9710). Figure 5C shows the box-plots of the cathodic currents at the different stages in the range between 0 to 600 mmHg, in which all animals were considered (n=6). In this case, hyperoxia vs. hypoxia showed an extremely significant difference (p=0.0003, one-way ANOVA), whereas hyperoxia vs. recovery demonstrated no significant difference (p=0.7258). In a similar manner, these results followed the same trend of those for pO_2 obtained with the EPOC® device for hyperoxia vs. hypoxia (p<0.0001) and hyperoxia vs. recovery (p=0.0453, one-way ANOVA). When the current range between 0 and 300 mmHg is graphed, a box-plot (Figure

5D) shows the current variation for the different stages and displays an extremely significant difference between hyperoxia and hypoxia (p<0.0001). While a significant difference (p=0.0107, one-way ANOVA) was found between hyperoxia and recovery, mainly due to the few data points collected for the latter stage.

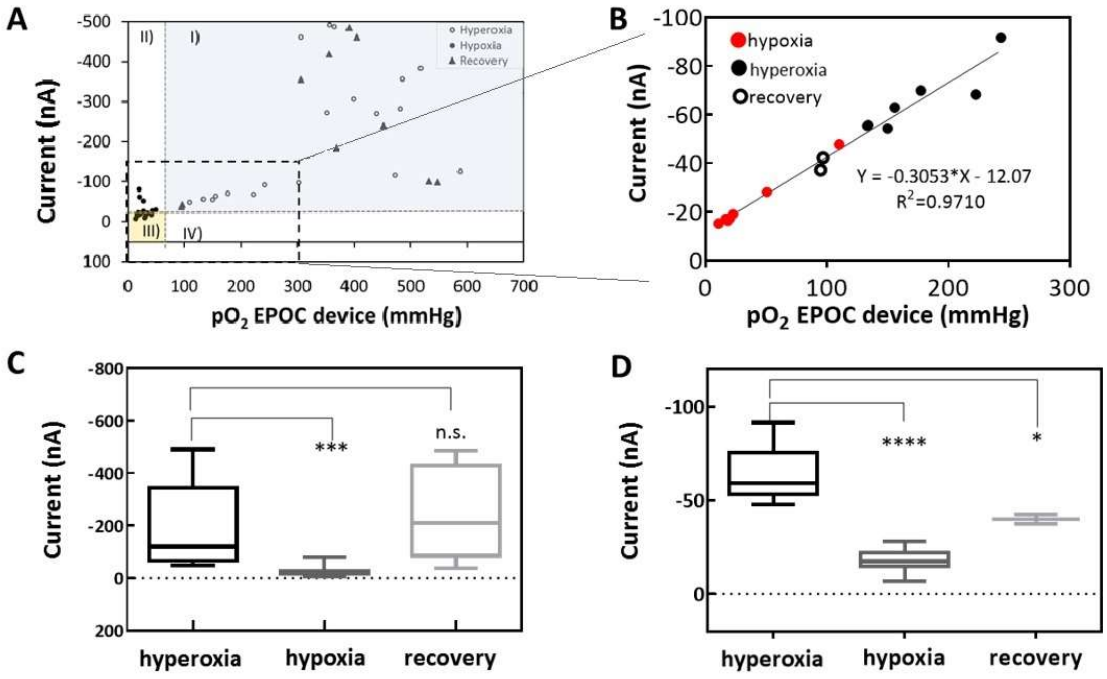

Figure 5. *ex-vivo* studies: (A) Correlation between cathodic current and pO_2 from EPOC® device in the range of 0-600 mmHg. Quadrants "I" and "III" shows hyperoxia and hypoxia zones, respectively; (B) Correlation between cathodic current a pO_2 from EPOC® device in the range 0-300 mmHg; (C) Box-plot of the current at different stages device in the range of 0-600 mmHg. (n=6 sensors, n=6 rabbits); (D) Box-plot of the current at different stages device in the range of 0-300 mmHg (n=2 sensors, n=2 rabbits)

It is worth mentioning that differences in sensitivity were observed between the *in-vitro* stored blood calibration and the *ex-vivo* experiments, being higher in *in-vitro* test. These differences are possibly due to the oxygen content for both systems. In the blood, the O_2 is carried as dissolved oxygen in a small fraction about 1-2%, and the remaining O_2 (98-99%) is combined with hemoglobin. Therefore, the content of O_2 in blood represents the amount of O_2 bound to hemoglobin (O_2 saturation and hemoglobin levels) and the amount of O_2 dissolved in arterial blood (*i.e.* pO_2) per 100 mL of blood.(Collins et al., 2015) In our experimental setup for *in-vitro* blood calibration, aged stored blood samples up to 1 week, were exposed for longer time to atmospheric oxygen (higher pO_2) during the measurements, resulting in a higher sensitivity

because more O_2 was dissolved and was more accessible for reduction than the O_2 bound to hemoglobin in blood *ex-vivo* samples.

On the other hand, during the mammalian respiration process, the pO_2 decays as it passes though the respiratory tract, then to the blood, and finally to the tissues.(West, 2016) Arterial pO_2 values for normoxic and hypoxic conditions in rabbits have been reported about *ca.* 110 mmHg and below 40 mmHg, respectively.(Sakai et al., 1999; Marriott and Marshall, 1990) In our *ex-vivo* studies arterial blood samples used were freshly extracted during hyperoxia or hypoxia induction and immediately analyzed. The current responses obtained in a range up to 300 mmHg were considerably lower than *in-vitro* blood studies and could be attributed to the pO_2 of those fresh blood samples. This would also explain the lower sensitivity of these investigations when comparing with *in-vitro* blood experiments.

3.3 in-vivo studies

Since there is no standard sensor for measuring pO_2 in tissue, no standard device was utilised for comparison of our *in-vivo* measurements. For this purpose, EPOC® device used for blood measurements was also employed for this comparison. However, it was not possible to obtain a good correlation between the current responses and the pO_2 in tissue, mainly because the obtained pO_2 at different states came from the arterial blood measured with the EPOC®, and not from the tissue itself. In consequence, the correlation current vs. pO_2 was not representative of the pO_2 changes occurred in tissue. Regardless, the results showed an extremely significant difference between hyperoxia and hypoxia in tissue (p<0.0001), as well as between hyperoxia and recovery (p=0.001), as illustrated in Figure 6. This meant that the developed sensor was able to successfully discriminate between high and low pO_2 levels in tissue. Nevertheless, no significant difference was observed between hypoxia and recovery (p=0,9118, one-way ANOVA). In contrast, for the *in-vivo* investigations, our O_2 sensor was not able to detect the increase of O_2 during the recovery phase. We hypothesize that after a prolonged period of hypoxia, the tissue is not capable to recover itself and, due to this, the recovery state was not detected in tissue. Moreover, when comparing with arterial O_2, which is in constant flow and renovation, the diffusion of O_2 is slower in tissue, taking longer time for reoxygenating it.

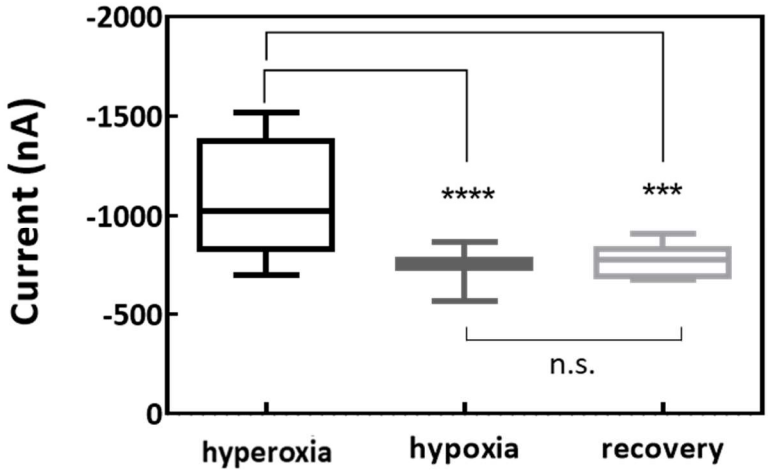

Figure 6. Box-plot of the correlation of cathodic current of experiment stages in *in-vivo* studies. *n*=7 sensors, *n*=6 rabbits

3.4 Pt-Nafion oxygen sensor reproducibility

The reproducibility of the Pt-Nafion oxygen sensor was evaluated by triplicate using different sensors for each study as stated in the Methods and Materials section. The relative standard deviation (RSD) varied between 3 and 8 % for the *in-vitro* analyses. However, for *ex-vivo* sensing, the RSD value reached up to 16 %, which was possibly due to the variability of the animals used in this study and, additionally to, the matrix effect.

4. Conclusions

In this work we have developed an electrochemical micro-sensor for oxygen detection in tissue (*in-vivo*) and arterial blood (*ex-vivo*). The *in-vitro* characterization in buffer of the Pt-Nafion® sensor showed a linear current response with excellent sensitivity of -2.4958 nA/mmHg (-1.495 nA/µM), resulting higher than similar Pt-based electrochemical sensors. *Ex-vivo* and *in-vivo* experiments were carried out in a hypoxic model in adult rabbits, by modulating the fraction of inspired O_2. Under different animal oxygen breathing stages, blood samples were extracted from the carotid artery and measurements were taken for *ex-vivo* purposes, while another implanted O_2 sensor was continuously monitoring inside muscular tissue. *Ex-vivo* studies in blood were successfully conducted and obtained a sensitivity of -0.305 nA/mmHg, following the same trend as the obtained with the standard EPOC®. Moreover, *in-vivo* studies showed that the Pt-Nafion® sensor was able to discriminate between hyperoxia and hypoxia states, despite the lack of standards for O_2 measurements directly in tissue.

However, this is still an ongoing project and improvement of certain aspects are still required. The fabrication of the micrometric sensors used for this project were manually produced, implying the potential for subsequent decrease of reproducibility between sensors. The fabrication needs to be industrially scalable for future commercialization of this technology. Moreover, the calibration of the sensors is not easy, since it is difficult to control the real concentration of O_2 adsorbed or desorbed in the solution. Although a parallel measurement with an optical device is performed, the response of the optical sensor is slower than that of the O_2 interchanging with the atmosphere. Furthermore, non- standard commercial device for tissue O_2 monitoring is available, being difficult to quantify our measurements in this matrix.

Overall, the developed microarray technology presented in this work would enable us to monitor changes of the oxygen content from any part of the tissue due to its small size, which currently is not possible, since no commercial devices are available for tissue ischemia monitoring. This type of tissue measurement is crucial for medical decision making in many different diseases such as stroke, compartmental syndrome, free-flaps reconstructive surgery, among others. Future research focused in the development of a telemetric system would give even more chances for its clinical applicability as it would allow real time and continuous monitoring.

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