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Brain tissue oxygen pressure monitoring using polyphenylenediamine-polyurethane-coated carbon fiber microelectrodes

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Letter

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Abstract

Brain tissue oxygen pressure (PbtO₂) monitoring is important to understand brain metabolism at rest or during neuronal activation. Electrochemistry provides high spatial and temporal resolution to achieve PbtO₂ monitoring, especially platinum (so-called Clark electrodes) and carbon fiber microelectrodes (CFMEs). However, a major problem with in vivo PbtO₂ recordings is a significant loss of sensitivity due to sensor fouling in the living brain tissue. Here, we tested a polyphenylenediamine-polyurethane (PPD-PU) coating to minimize electrode fouling in vivo and improve the stability of PbtO₂ recordings. PPD and PU coatings were deposited on CFMEs (7 μm diameter, 100 μm long, 4400 μm²) and displayed less fouling in vivo than bare CFMEs, or CFMEs coated with PPD only. Oxygen reduction started around -300 mV vs Ag/AgCl and PPD-PU deposition increased microelectrode diameter by 1-2 μm. Carbon fiber microelectrodes coated with PPD-PU were used for 2.5-5 h of PbtO₂ monitoring in anesthetized rats and there was no significant change in O₂ sensitivity before and after in vivo implantation. These results indicate that PbtO₂ monitoring can be achieved reliably using PPD-PU coating on carbon fiber microelectrodes, preventing electrode fouling in vivo, and providing an inexpensive and minimally invasive tool to study brain oxygen metabolism.

Introduction

Oxygen is a vital energy metabolite on which the brain, and all other living tissues, is highly dependent. Brain tissue oxygen pressure (P_{btO_2}) is therefore a crucial parameter to monitor and understand brain patho-physiology. Brain tissue oxygenation can be estimated non-invasively, using the blood-oxygen-level-dependent (BOLD) signal in magnetic resonance imaging (MRI) ¹, or the near-infrared spectroscopic (NIRS) signal². However, these non-invasive signals reflect the percentage of oxygenated hemoglobin in blood and are therefore only qualitative. So far, the only methods to estimate P_{btO_2} quantitatively, are electrochemical methods. With these methods, a microelectrode is inserted in the brain tissue and polarized to trigger O_2 reduction. The measured reduction current can then be related to the local O_2 concentration by calibration. Although simple in principle, this technique faces two major challenges. First, the implanted microelectrodes must be extremely small to avoid damaging local microvasculature, and hence, the local O_2 supply to avoid monitoring non-physiological phenomenon. The average distance between blood vessels estimated around $25\ \mu\text{m}$ ³ stands as an upper limit for microelectrode diameter. Second, the sensitivity and specificity of the O_2 sensors must remain stable during the in vivo monitoring period, which is evaluated by the comparison of pre- and post-calibration values. However, in most implanted sensors, the sensitivity to O_2 decreases after a period of in vivo implantation due to biofouling.

Sensor biofouling is a process that starts with contact of the sensor with the living brain tissue, when proteins and immune cells adhere to the surface of the electrode or to its outer membrane if present⁴. Protection against biofouling by the living brain tissue is a difficult task because of the sheer number of adsorbing molecules in this matrix. In the context of biofouling, recessed microelectrodes are among the most stable O_2 detecting devices. These electrodes are composed of an electroactive material, usually platinum, which is inserted in a glass pipette with a small opening that is closed by a polymer membrane to restrict the diffusion of all molecules larger than O_2 . Leland Clark was the first to use a recessed electrode for O_2 detection, where a platinum cathode was insulated with glass containing a $3\ \mu\text{m}$ opening for analyte diffusion⁵. Other variations of this electrode design have been proposed subsequently⁶ and commercial oxygen microsensors based on this principle are now available for various applications including brain monitoring in laboratory animals⁷. Despite their excellent performance and utility, these recessed electrodes are expensive, fragile, and are difficult to

fabricate and miniaturize, which is particularly problematic for recordings in freely moving animals such as laboratory rodents, necessitating a simpler microelectrode solution.

Simpler, less-expensive O₂ detecting devices have been proposed, with carbon fiber⁸, platinum wire^{9, 10} or silver wire/carbon paste¹¹ microelectrodes, but these microelectrodes suffer from significant biofouling by brain or blood proteins, and immune reactions^{4, 12}. These electrode designs are subject to significant biofouling compared to the Clark style recessed electrode because of direct exposure of the electrode surface to the chemical matrix. To counteract this phenomenon, a variety of coatings have been proposed, such as Nafion^{8, 13}, cellulose nitrate (collodion)¹¹, or polyethylene¹⁰, but their protective effects are either partial or not very reproducible. Here, we propose a novel coating paradigm for carbon fiber microelectrodes (CFMEs) to minimize *in vivo* biofouling using electropolymerized poly-phenylenediamine (PPD) followed by dip-coating of polyurethane (PU). PPD has been previously observed to form a size-exclusion screening layer to prevent the non-specific oxidation of redox active molecules by microelectrode biosensors *in vivo*^{14, 15}. More specifically, PPD has been used to protect nitric oxide sensors implanted in the rat brain¹⁶. PU coatings display high biocompatibility and have been used to increase the linear range (*via* limitation of the rate of diffusion) and lifetime of glucose biosensors^{17, 18}. We hypothesized that the combined use of PPD and PU could provide a biocompatible screening layer capable of blocking the adsorption of macromolecules and immune cells, and protect CFMEs from biofouling, providing a stable microelectrode substrate to monitor PO₂ in the living brain.

Cortical spreading depolarization (SD) is a pathological phenomenon that manifests as a wave of mass neuronal depolarization that propagates across the brain. SDs are characterized by near-complete depolarization of neuronal and glial cells¹⁹. This results in an efflux of intracellular potassium²⁰ and other molecules such as glutamate²⁰, creating a reactive chemical microenvironment. The large-scale disruption of cell membrane polarization characteristic to SDs is accompanied by a distinct and sharp decrease in local PO₂ caused by an increase in energy demand as the brain tissue repolarizes, and this has been observed by electrochemical techniques²¹. In this work, we evaluate the stability of our novel coating while comparing the performance of our

CFMEs to the commercial standard under the highly dynamic chemical conditions of a spreading depolarization.

Experimental section

All chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Phosphate buffered saline (PBS) was prepared at 0.01 M, pH 7.4. CFMEs were fabricated using 7 μm diameter carbon fibers (Goodfellow, Lille, France) protruding out of a borosilicate micropipette (Multichannel Systems, Reutlingen, Germany, 1.5 mm OD, 0.86 mm ID) by 150 μm (exposed carbon surface was 6,600 μm^2). PPD was deposited by electropolymerization in a 100 mM m-phenylenediamine solution in PBS, at +700 mV vs Ag/AgCl pseudo reference. PU was deposited by two dip-coatings in a 5% PU solution in cyclohexanone-ethanol (1:1) and was allowed to evaporate for 5 minutes per coating. *In vitro* calibrations were performed in PBS at 37°C. Cyclic voltammetry was performed between -500 mV and +600 mV vs Ag/AgCl pseudo reference at 20 $\text{mV}\cdot\text{s}^{-1}$ using a two-electrode potentiostat. Only CFMEs displaying more than 5 pA/mmHg were selected for the study, which represented 29/35 electrodes (65%).

Animal experimental protocols were approved by the CELYNE (CEA-042) committee on animals in research and registered by the French Ministry of Research under reference APAFIS #28812-2020121816471562 v10. All animals were treated according to the European Directive 2010/63/UE and experiments respected the ARRIVE guidelines. *In vivo* monitoring was performed in 10-15 weeks old male and female Sprague Dawley rats (Envigo, Gannat, France). Animals were accommodated in cages of 2 or 3 with food and water ad libitum and exposure to a 12hr:12hr light-dark cycle in the Central Animal Facility of the Lyon Neuroscience Research Center. Animals were anesthetized with isoflurane anesthesia (5% induction, 1.2-1.5% maintenance) in 30% oxygen with air. A subcutaneous injection of buprenorphine (BUPAQ®, 0.05 mg/kg) was performed 20 min before incision. Oxygen saturation was monitored at the paw and maintained above 90%, while temperature was kept at 37°C with a rectal probe and thermal pad. The scalp was then incised, and the surface of

the skull cleaned with a cotton swab and saline. To expose the dura mater, a 5 mm diameter circular craniotomy was performed over the parietal cortex for monitoring PbtO₂ (3,8 mm posterior to bregma, 3 mm lateral), and a smaller 2-3 mm opening was prepared anterior to bregma for the induction of depolarizations. The dura was resected to expose the pia mater at the monitoring window and rinsed with artificial cerebrospinal fluid (aCSF). The PbtO₂ sensor was a home-made bare CFME or PPD-PU-coated CFME, or a commercial clark-type O₂ microbiosensor (5µm tip, OX-10, Unisense), and was implanted 0.9 mm deep and left undisturbed for 30 min before monitoring began. Cortical spreading depolarization was triggered by mechanical pressure on the dura at the level of the anterior cranial window. Until the concentration level recovers to a stable state, the monitoring was then stopped.

Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical analyses were performed using a paired student t test or ANOVA followed by Tukey's post-hoc test for normally distributed data (normality was confirmed by the Kolmogorov-Smirnov test for all data groups). Significance was set at p<0.05.

Results and discussion

Comparison of CFMEs Before and After Implantation In Vivo

Taking advantage of a series of *in vivo* experiments studying cortical spreading depolarizations (SDs) in rats, we tested several types of microelectrodes for monitoring PbtO₂. For these tests, the total implantation time *in vivo* was 2h to 5h. Preliminary experiments on platinum wire microelectrodes revealed a large loss of sensitivity between pre- and post-implantation calibration values, which suggests that electrode fouling occurred *in vivo*. The loss of sensitivity was -68 ±9 % for bare platinum wire microelectrodes, and -53 ±10 % for platinum wire coated with PPD and PU (n=2). These preliminary tests reveal significant biofouling of platinum microelectrodes exposed to blood or albumin previously observed by others²², and led us to test our coating paradigm with other electrode materials, such as carbon fiber (CF). CFMEs are inexpensive to produce and are easily modified with protective coatings. Our initial experiments demonstrate that the CFMEs suffered a significant -85 ±3 % loss of sensitivity for bare CFMEs (p=0.019, paired student t test, n=6). However, this loss in

sensitivity to O₂ was decreased when the carbon fiber was coated with PPD (-36 ±9 %, p=0.03, paired student t test, n=9), and nearly eliminated when the carbon fiber was coated with PPD and an additional layer of PU (-2 ±7 %, p=0.91 NS, paired student t test, n=5, Fig.1A). These data indicate that electrode fouling was significantly mitigated by coating with PPD and PU. Consequently, sensitivity to O₂ after *in vivo* implantation was maximal with PPD and PU coating (15 ±3 pA/mmHg), intermediate with PPD coating only (5.9 ±0.7 pA/mmHg) and minimal with bare CFMEs (4.2 ±1.0 pA/mmHg, Fig.1). An ANOVA performed on the three coating paradigms explored in this study revealed a significant effect of microelectrode coating on post-implantation PO₂ sensitivity (F(17,2)=7.12, p=0.006). Tukey's post hoc test revealed a significant difference between the sensitivity of bare CFMEs and that of CFME coated with PPD and PU (p=0.007), and a significant difference between CFMEs coated with PPD and PU, and those coated with PPD only (p=0.014, Fig.1B).

Surface Characterization of CFMEs

We then focused on characterizing the CFMEs coated with PPD and PU with scanning electron microscopy, which revealed a uniform coating 0.5 to 1 μm thickness (Fig. 2A, B, bare CFME, 2D, E, CFME coated with PPD and PU). Cyclic voltammetry from -500 mV to +600 mV vs Ag/AgCl revealed O₂ reduction starting at -300 mV and oxidation above 550 mV. Interestingly, PPD and PU coating did not change the potential at which O₂ reduction and oxidation were detected, suggesting that coating with PPD and PU is uniquely a steric barrier that does not alter the electrode surface. Moreover, charging currents on the voltammograms were increased after PPD and PU, indicating larger capacitance due to the addition of a nonconductive polymer layer around the CFME. Overall, these analyses are consistent with the PPD and PU coating behaving as a screening layer protecting the CFME against biofouling, while permitting O₂ redox reactions to occur unchanged. PPD or PU have already been used for coating microelectrodes for brain monitoring. PU is often used for coating enzyme biosensors to limit their dynamic range by restricting the diffusion of the enzyme substrate while leaving O₂ diffusion unchanged^{18, 23, 24}. Similarly, PPD, an electropolymerized coating that is often used to prevent non-specific oxidation of endogenous redox-active molecules in the brain, can block the diffusion of biogenic amines like dopamine or serotonin, but not O₂ or H₂O₂^{14, 18, 24}. There is therefore converging evidence that both PPD and PU can act synergistically to offer an improved protective steric barrier for

CFMEs that does not interfere with O₂ diffusion and redox reactions at the microelectrode.

In vivo comparison of CFMEs with Novel Coating to Commercial Standard

Next, the performance of bare CFMEs for PbtO₂ monitoring was compared to that of CFMEs coated with PPD and PU in anesthetized rats following the induction of a cortical SD. SDs induce a sudden drop in PbtO₂ due to the increased O₂ consumption related to oxidative glycolysis during brain tissue repolarization²¹. This sudden decrease in PbtO₂ is followed by a subsequent increase in local vasodilation to allow more O₂ diffusion into the brain tissue. Despite slight differences in shape between PbtO₂ recordings using bare or PPD-PU-coated CFMEs, our *in vivo* recordings displayed this biphasic shape (Fig.3). As expected from the significant loss of sensitivity due to biofouling, revealed by pre- and post-calibration values, O₂ reduction currents detected in basal conditions were smaller with bare CFMEs (Fig.3A) compared to CFMEs coated with PPD and PU (Fig.3B, 0.43 ±0.12 nA vs 1.12 ±0.25 nA, p=0.028), providing enhanced signal to noise ratio for PbtO₂ detection *in vivo*. In addition, because CFMEs coated with PPD and PU had similar pre- and post-calibration values, it was possible to predict their sensitivity *in vivo*, and thus provide quantitative estimates of PbtO₂ values during SDs. Furthermore, PbtO₂ estimates collected with the commercial recessed electrodes from Unisense found similar resting PbtO₂ levels (19.1 ±2.1 mmHg PPD-PU CFEMs, 19.0 ±1.3 mmHg Unisense, p>0.99) and patterns of PbtO₂ variations evoked by SD (Fig.3C). The PbtO₂ levels between our dual-coated sensor and the commercial sensor were not significantly different 11s after SD initiation (6.1 ±2.3 mmHg PPD-PU CFEMs, 6.6 ±2.1 mmHg Unisense, p>0.99), or 10 min after SD (14.2 ±2.3 mmHg PPD-PU CFEMs, 16.8 ±2.0 mmHg Unisense, p=0.94). Additionally, the mean level of PO₂ measured by CFMEs coated with PPD and PU slightly decreased after SD, but a similar decrease was detected by commercial sensors, suggesting a physiological phenomenon.

Discussion

The comparison of pre- and post-calibration values is critical to the quantification of *in vivo* concentrations because the electrode sensitivity must remain stable to produce reliable measurements. If pre and post calibration parameters are not significantly different, it may be assumed that the sensor has not undergone mechanisms of fouling

that would impact the validity of the quantified concentrations. The mechanisms of microelectrode fouling have been categorized into two major processes. The first is biofouling, occurring *in vivo*, where proteins such as albumin and/or blood cells and platelets adsorb to the surface of the sensor and prevent proper access of the analytes of interest to the electrode^{4, 22, 25}. Chemical fouling is the second relevant process, where products of the electrochemical reaction of interest irreversibly adhere to the electrode surface⁴. Both mechanisms result in significantly lower sensitivity that is usually not reversible. CFMEs cannot be rescued post-fouling (without surface etching) due their hydrophobic surface, which readily interacts with other hydrophobic molecules *in vivo* under the thermodynamically favorable condition of an aqueous environment. Previous experiments have reported that other types of sensors that failed *in vivo* could regain their sensitivity to varying degrees after storage in saline solution²³. However, in this case it was the enzyme membrane on a biosensor that was fouled, not the electrode itself. Additionally, other sources have indicated that fouling agents that adhere via hydrophilic interactions are more likely to be reversible in aqueous solutions²⁶. With enzyme biosensors, it is probable that the hydrophilic residues in the enzyme layer underwent electrostatic interactions with hydrophilic molecules *in vivo*, which were liberated by the saline solution, thereby refreshing the enzyme membrane. Thus, in certain cases, fouling processes happening *in vivo* can be reversed, though this has never been observed for our sensing paradigm (oxygen sensing via CFMEs), therefore not significantly different pre- and post-calibration values can reasonably be taken as an indication that biofouling is minimal. Finally, the similarity between the PO₂ values obtained with our CFMEs coated with PPD and PU and with the commercial standard (which is not prone to fouling), is additional evidence that our quantitative PO₂ estimates are not distorted by biofouling.

In addition to biofouling by macromolecules and cells, a number of small molecules have also been described as potential biofouling agents, such as the antidepressive drugs fluoxetine, escitalopram, and sertraline²⁷, or serotonin and its oxidation products²⁸. In this regard, our first selected coating, PPD, provides a tight screening layer blocking more than 95% of ascorbate oxidation as well as that of other redox active molecules of similar size^{15, 29}. We propose that the second layer of PU stabilizes the PPD layer, which may degrade over time, especially *in vivo*. Because of its extremely low molecular weight, O₂ can still diffuse through this screening polymeric

layer, whereas larger biofouling molecules are blocked. To our knowledge, no simple molecule the size of O_2 has been described to foul bioelectrode surfaces. Our results provide evidence in favor of coating the microelectrode with polymers that block large molecules at a distance from the sensing surface to monitor $PbtO_2$, and more specifically dual coating of PPD and PU to provide an improved barrier against biofouling.

Conclusion

CFMEs coated with PPD and PU are a low cost, minimally invasive strategy for monitoring $PbtO_2$ while preventing biofouling by macromolecules and immune cells present *in vivo*. These sensors were tested for a few hours in anesthetized animals, attesting to their stability. Future studies involving longer implantation times in freely-moving awake animals are now warranted to estimate their maximal lifetime in the brain of a living animal.

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Figure legends

Figure 1: Sensitivity of different types of oxygen sensors based on carbon fiber microelectrodes (CFME) types: bare CFME (n=6), CFME coated with electropolymerized poly-phenylene diamine (PPD, n=9), and CFME coated with PPD and polyurethane (PU, n=5). Sensitivity was measured at the end of a 2-2.5 h in vivo monitoring of a cortical spreading depolarization in an anesthetized rat. Although all sensor types had a similar sensitivity prior to in vivo implantation, CFMEs coated with PPD and PU were significantly more sensitive to oxygen than CFMEs coated with PPD only ($p=0.014$) and bare CFMEs ($p=0.007$) after in vivo monitoring, revealing reduced electrode fouling with the PPD and PU coating.

Figure 2: Scanning electron microscopic images of a bare carbon fiber microelectrode (A, B) with its cyclic voltammogram in phosphate buffered saline (PBS) in equilibrium with air (red trace) or nitrogen (black trace, C). Similar microscopic pictures (D, E) and cyclic voltammograms (F) for a poly-phenylenediamine and polyurethane-coated carbon fiber microelectrode. Scale bars are 50 μm (A and D) and 5 μm (B and E). Cyclic voltammetry was performed between -500 and +600 mV vs Ag/AgCl at 20 $\text{mV}\cdot\text{s}^{-1}$ in PBS 0.01 M pH 7.4.

Figure 3: Example of PO_2 monitoring with bare CFMEs (A, n=5), CFMEs coated with PPD and PU (B, n=5), and commercial PO_2 sensors from Unisense (C, n=6). CFMEs coated with PPD and PU detected larger oxygen reduction currents. Most importantly, because the sensitivity was not significantly different before and after in vivo implantation, it was possible to quantify PbtO_2 detected by CFMEs coated with PPD and PU. PO_2 estimates by PPD-PU CFMEs were similar to those by commercial

PO₂ sensors. Absolute oxygen reduction currents are shown in black (gray shading for SEM), and PO₂ estimates are shown in red (pink shading for SEM).

Figure 1

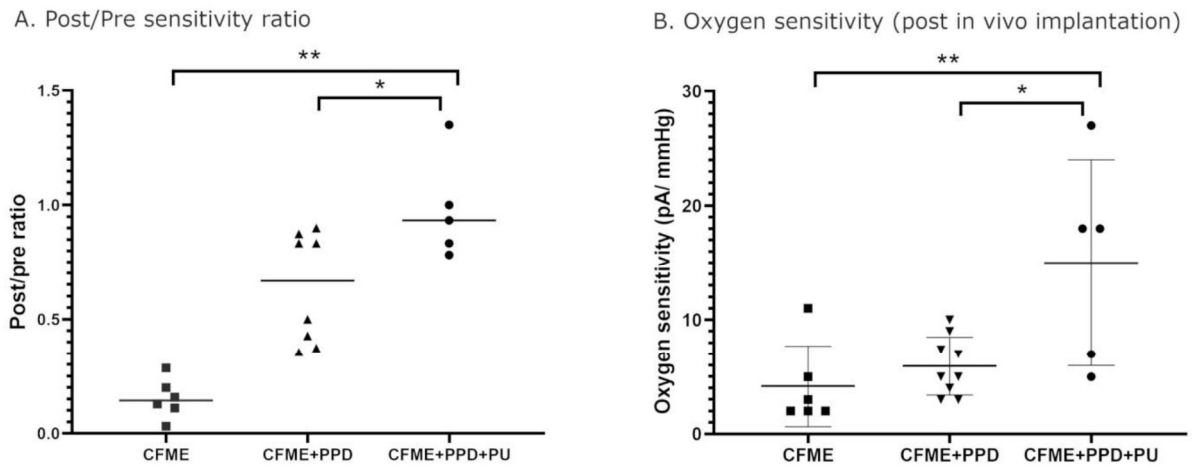


Figure 2

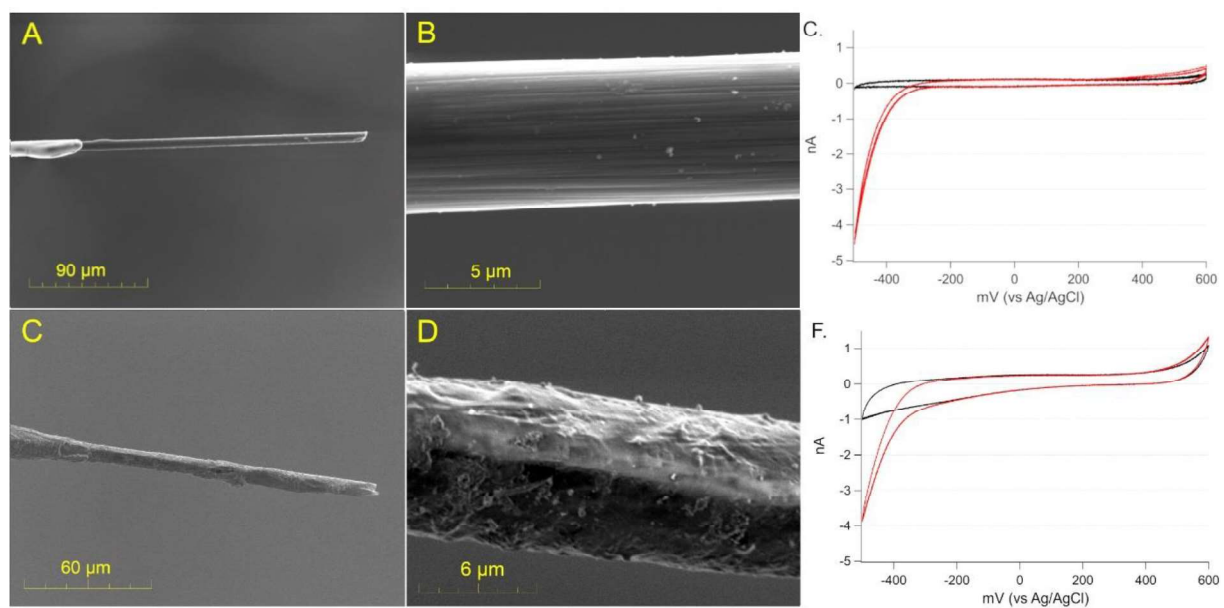
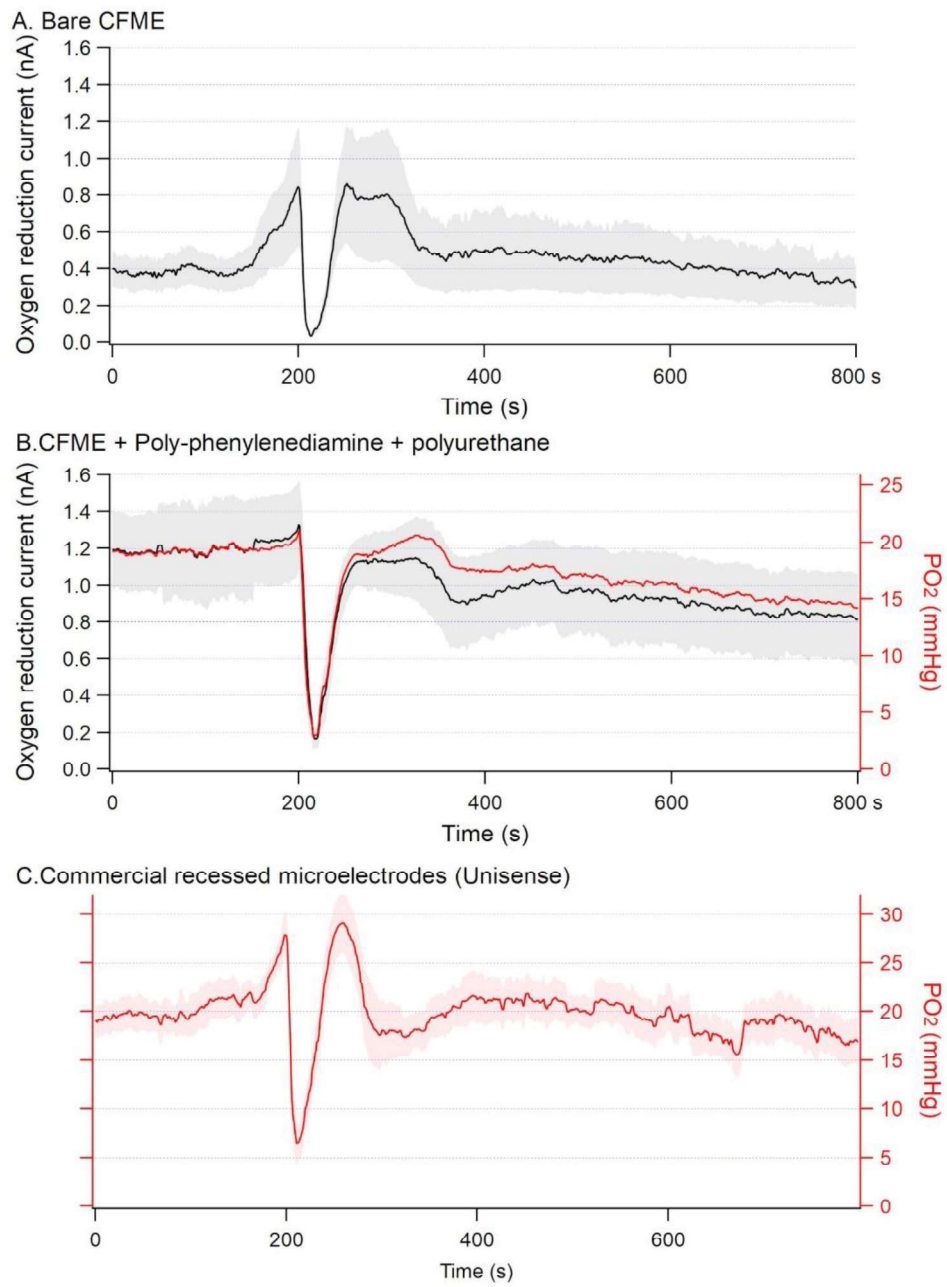


Figure 3



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