Electrochemical Methods and Sensors for Real Time Monitoring of Oxidative Stress Biomarkers

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Abstract. With the increasing evidence of the involvement of oxidative stress in many diseases, interest in developing methods that can measure oxidative stress biomarkers in real time has continuously increased. Electrochemical methods and sensors are ideally suited for monitoring reactive oxygen (ROS) and nitrogen (RNS) with very high sensitivity and real time resolution, proving the measurements needs to quantify these short lived reactive species in situ. This chapter describes the development status of electrochemical methods, sensors and biosensors for *in vivo/in vitro* monitoring of ROS/RNS and provides examples of measurement capabilities in pathophysiological conditions associated with oxidative stress for disease monitoring, pharmacological screening and other relevant biomedical applications. Recently reported microelectrode designs and methodologies based on chemical and enzyme-based approaches as well as their advantages and limitations are discussed. Examples of possible implementation and translation of this technology to evaluate oxidative stress in cellular, tissues and whole animal models are also provided to demonstrate potential of this technology in biology and biomedical fields.

Keywords: Electrochemical sensors, biosensors, ROS/RNS, measurements, microelectrodes.

1. Introduction

In this chapter we describe the principle, performance and application of electrochemical methods and sensors for the detection of oxidative stress biomarkers, focussing on reactive oxygen and nitrogen species (ROS and RNS), formed by reactions involving oxygen or nitrogen species. These include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), hydroxyl ($HO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), hypochlorous acid/hypochlorite (HOCl/$^{-}$OCl) and singlet oxygen ($^1O_2$) [1, 2], as well as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) as representative RNS. These species are produced during cellular processes such as oxidative phosphorylation, catabolism, phagocytosis and have different life-time, bioactivity, and cellular function [3, 4]. ROS/ are responsible for the overall oxidative load in biological systems [2, 4]. In normal physiological conditions, enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (e.g., glutathione, thioredoxin, ascorbate) antioxidants regulate oxidative levels and prevent oxidative overload [5, 6]. ROS is known to play a critical role in redox signalling, allowing cells to adapt to environmental changes [3, 5, 7]. However, when these species are present in excess, they can damage cells, proteins and DNA, and lead to an overall state of oxidative stress [8]. This redox imbalance is contributing to the development and progression of a myriad of diseases [9, 10], and if not corrected could result in severe conditions, including organ failure [11-13]. Because ROS/RNS are highly reactive and short lived, with lifetimes as low as in the ns, their direct measurement in cells, tissues and organisms is extremely difficult. Therefore, in order to fully understand the contribution of ROS/RNS and the relationship between oxidative stress in physiological and pathological conditions [14, 15], there is a need to develop analytical methods and tools that can monitor ROS/RNS in real time and in situ, and investigate disease mechanisms and pharmacological efficacy. Their accurate measurements in situ could contribute to the understanding of their physiological functions.

Conventional methods to measure ROS/RNS include electron paramagnetic resonance and photon emission spectroscopy [16, 17] and fluorescence spectroscopy with ROS/RNS-specific dyes [18]. These methods have limited ability to measure these species in biological tissues and do not provide accurate quantification of the level of these species in biological samples. Due to the high sensitivity and the ability to fabricate small probes that can be

inserted in tissues and be used in cellular cultures, electrochemistry provides unique opportunities to detect ROS/RNS release in real time and with high spatial resolution [19-23]. Many different configurations of electrochemical sensors and detection methods have been reported in literature, with the most common examples for the detection of species such as H_2O_2, NO and $ONOO^-$ and $O_2^{\cdot}$ [24]. This chapter describes the functioning and provides several examples of electrochemical microbiosensors for ROS/RNS measurements in biological systems. We also provide a discussion of the remaining challenges and translational aspects of this technology for biology and biomedical applications.

2. Electrochemical properties of ROS/RNS

ROS/RNS have characteristic redox potentials enabling measurement by electrochemical means. For example, the reduction potential of, and $O_2^{\cdot}$ to H_2O_2 is~0.94V, while that of nitric oxide (NO) as a representative RNS is ~ +0.8V depending on the electrode and electrolyte conditions. Table 1 provides representative examples of redox reactions and oxidation potentials of common reactive species.

Table I. Electrode Reactions and Standard Potentials for Powerful Oxidants Formed in Aqueous Sulfate and Chloride Solutions [25]

Oxidant	Reaction	E^o, V vs. NHE	Ref.
ozonide radical anion	$O^{\bullet-}_{3(aq)} + 6H^+ + 5e = 3H_2O$	1.575	a
hydroxyl radical	$OH^{\bullet}_{(g)} + H^+ + e = H_2O$ $OH^{\bullet}_{(aq)} + H^+ + e = H_2O$ $O^{\bullet-}_{(aq)} + 2H^+ + e = H_2O$	2.82 2.722 3.426	g b c
atomic oxygen	$O_{(g)} + 2H^+ + e = H_2O$	2.421	[26]
ozone	$O_{3(g)} + 2H^+ + 2e = O_2 + H_2O$ $O_{3(g)} + 6H^+ + 6e = 3H_2O$	2.076 1.511	[26]
hydrogen peroxyl radical	$HO_2^{\bullet} + 3H^+ + 3e = 2H_2O$	1.684	h
hydrogen peroxide	$H_2O_2 + 2H^+ + 2e = 2H_2O$ $HO_2^- + 3H^+ + 2e = 2H_2O$	1.776 2.119	[26]
oxygen	$O_2 + 4H^+ + 4e = 2H_2O$	1.228	[26]
atomic chlorine	$Cl^{\bullet} + e = Cl^-$	2.410	[27, 28],d
dichlorine radical anion	$Cl_2^{\bullet-} + e = 2Cl^-$	2.090	[27]
hydroxyl chloride radical anion	$ClOH^{\bullet-} + H^+ + e = Cl^- + H_2O$ $ClOH^{\bullet-} + e = Cl^- + OH^-$	2.729 1.90	e [27]
chlorine monoxide	$Cl_2O + 2H^+ + 2e = 2Cl^- + H_2O$	2.153	[26]
chlorine dioxide	$ClO_2 + 4H^+ + 5e = Cl^- + 2H_2O$	1.511	[26]
hypochlorous acid	$HClO + H^+ + e = Cl^- + H_2O$ $ClO^- + 2H^+ + e = Cl^- + H_2O$	1.494 1.715	[26]
chlorine	$Cl_2 + 2e = 2Cl^-$	1.395	[26]
persulfate radical	$HSO_4^{\bullet} + e = HSO_4^-$	2.60	[29]
peroxysulfate	$S_2O_8^{-2} + 2e = 2SO_4^{-2}$	1.940	[29],f

a - on the basis of $\Delta G^0_f(O_3^{\bullet-}(aq)) = 11.25$ obtained from $E^0(O_2/O_3^{\bullet-})$ [30];

b - using $\Delta G(OH^{\bullet}_{aq}) = +6.0$ [28]; Koppenol-Liebman [30] obtained 2.59 V; calcd from Pourbaix data [26]: 2.832 V;

c - on the basis of pK of $OH^{\bullet}$: pK = 11.9 [53]; $E^0 = 3.308$ calcd from [30];

d - Berdnikov and Bazhin [31] obtained 2.55 V; Gratzel: 2.55 V [29];

e - using $\Delta G(ClOH^{\bullet-}_{aq}) = -25.18$ kcal/mol obtained from data of Ref. [27];

f - 2.01 V given by Pourbaix [26];

g - from [32, 33];

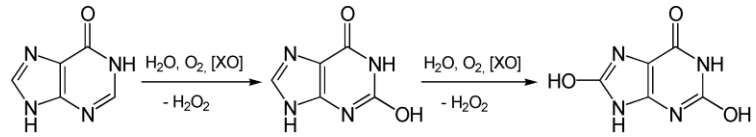
h - from [26].

Nitric oxide (NO), as a representative RNS, is a diffusible gaseous radical with a 1-10 second biological half-life playing an essential physiological regulator in immunology and cell signaling [34, 35]. Depending on the pH and the environment. NO can be found in equilibrium with nitrite, (NO_2) and dinitrogen oxide (N_2O) [36], and under oxygen conditions can forms highly reactive peroxynitrate, $ONOO^-$, which is known to react with tyrosine residues in protein phosphorylation [37]. Superoxide ($O_2^{\cdot}$) is a by-product of mitochondrial

respiration [38] and known to be involved in redox regulatory signaling [39, 40]. $O_2^{\cdot}$ can induce formation of more toxic radicals such as hydroxyl radicals, that can damage cells, DNA and intracellular molecules, following mechanism such as shown in Equation 1:

$$O_2^{\cdot -} + H_2O_2 \rightharpoonup O_2 + OH^- + OH^{\cdot} \qquad \text{[Equation 1]}$$

It is well known that superoxide dismutase converts $O_2^{\cdot}$ superoxide to H_2O_2 hydrogen peroxide [41, 42] which can be assayed, and used to estimate the amount and rate of production of $O_2^{\cdot}$. H_2O_2 can also result from the degradation of adenosine monophosphate (AMP), which is converted to hypoxanthine and then further oxidzed to xanthine and uric acid by an enzyme xanthine oxidase (XO), as shown in Equation 2. H_2O_2 is also present in organelles, peroxisomes, during the reduction of dioxygen with fatty acids, polyamines, and other compounds, catalyzed by flavin adenine dinucleotide (FAD).



hypoxanthine xanthine uric acid

[Equation 2]

3. Electrochemical sensors and biosensors for ROS/RNS detection

Electrochemistry provides a valuable tool to for the quantification of ROS/RNS species in biological settings. Using small size microelectrode, specially designed probes coated with ROS/RNS specific receptors and coatings can be used to measure reactive species directly in biological media, cell populations, be inserted in tissues, measure single cell activity, and ultimately inside live cells [43]. ROS/RNS detection in biological models has been reviewed [19, 44, 45], but only few reported studies demonstrate measurements *in vivo* due to issues such as short lifetime, calibration drift, cross-reactivity, and interferences.

3.1. Materials and fabrication of electrochemical (bio)sensors

While many studies demonstrates electrochemical measurements of ROS/RNS, in most cases, the probes used have micro-size dimensions such as glassy carbon electrodes (GCE) which have restricted usability for cells or live tissues work. Therefore for these to be used in these

environments, electrode probes need to be designed to match the biological model of study to minimize perturbation of the environment. Parameters such as electrode size, coatings and morphology play an important role. Most microelectrodes are fabricated from carbon fiber microelectrodes (CFMEs) using a capillary puller. These CFME have sizes ranging from 5-10 □m. Gold or platinum wire microelectrodes with diameters of ~100 □m are also frequently used, particularly for measurements of $O_2^{\cdot}$ that involve bioimmobilization of biological receptors [46]. Efforts to develop miniaturized probes [47] involved procedures such as: flame-etching which was shown to form micrometer sized carbon fibers with diameter of 50-100 nm at the top and a length of 30-100 μm [48], electrochemical pre-treatment [49], pyrolitic deposition of carbon within pre-pulled nanopipettes with diameter varying in the range 5-200 nm [50], or the use of a 3D printed mold as shown **in Figure 1** [51], a procedure enabling high volume production.

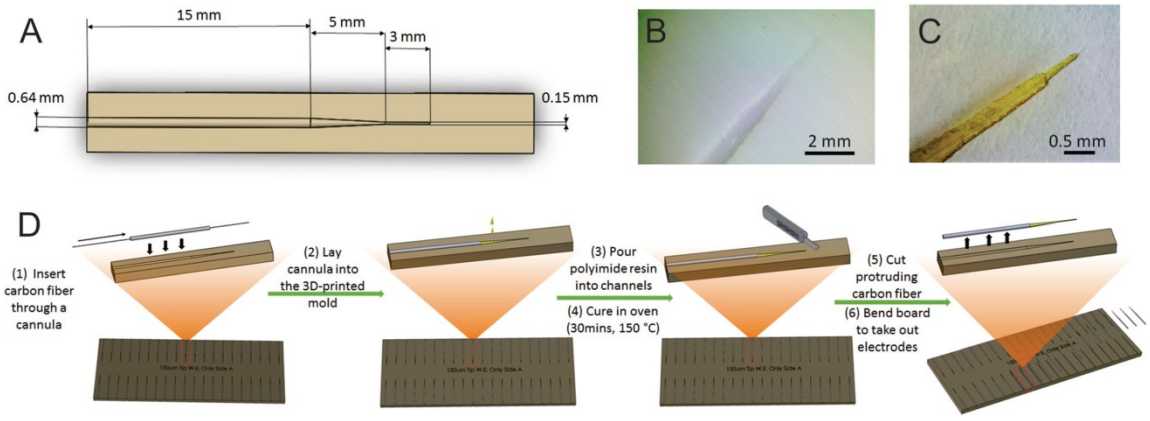


Figure 1. Example of fabrication process of CFMEs using 3D-printed mold on polyimide: (A) Close-up of the design of one channel for 3D-printed microelectrode fabrication. (B) Image of the tip from a 3D-printed mold, scale bar: 2 mm. (C) Image of a polyimide-insulated carbon fiber microelectrode fabricated using the mold, scale bar: 0.5 mm. (D) Scheme of the process of microelectrode fabrication. (Reprinted with permission from[51].

To achieve sensitive and selective measurements, electrodes are functionalized with chemical or biological coatings. ROS/RNS can be measured directly at their characteristic potentials, such as measurements of NO oxidation at ~ 0.8 V vs Ag/AgCl, or, the oxygen/superoxide redox couple at ~-0.33 V vs NHE [22]. Used of platinized carbon MEs (~10 □m diameter)

was demonstrated to be particularly suitable for single cells measurements [22, 52-54] and enable studies of species inside single phagolysosomes of living macrophages by chronoamperometry [54]. Commonly, MEs are functionalized with catalytic materials and nanomaterials to enhance the sensitivity and selectivity. Carbon nanomaterials such as carbon nanotubes [55] or graphene [56] are the most commonly used due to their high surface area and conductivity, facilitating MEs with increased electrochemically active surface [57, 58] and improving detection performance for example for H_2O_2 [59]. Catalytic materials based on metallic layers or polymerized electrocatalytic mediators are also common. Examples include modification, some in composites made with graphene, Prussian Blue [60] or hemin [61, 62]. Bimetallic [63], metal-polymer [64] or metal-metal oxides [65, 66] are common examples of electrocatalytic materials for H_2O_2 detection. For example, nanoCuO nanostructures improved performance of H_2O_2 sensing [67], which can be used to develop miniaturized sensors [68]. Common electrocatalytic mediators are materials such as Prussian Blue [69] and Meldola Blue [70, 71].

Because of the complexity of the biological environment and the presence of redox active compounds, such as uric acid and ascorbic acid in the media, a common difficulty for translating their use in 'real' environments is the issue of interferences [23]. To address this issue, MEs are chemically coated with perm-selective or electrostatic layers to reject interfering species through either size or charge. The use of materials such as Nafion and chitosan [72] have been shown to be effective at removing ascorbic acid interferences. Another problem is biofouling due to protein adsorption or deposition of reaction by-products. Some studies have shown that the preparation of CFMEs with carbon nanotubes improves the resistance to fouling [73].

Biological sensors or biosensors are protein-functionalized electrodes that contain a biological recognition layer, typically a redox protein, immobilized at surface of the electrode to specifically recognize the targeted species and translate recognition into an electrochemical signal. For example, cytochrome c (Cyt C) provide biomolecular recognition for $O_2^{\cdot -}$ through an electron transfer process at the surface of a MEs [74]. To fabricate the biosensor, Cyt C is immobilized onto the surface of a gold microwire and the immobilized layer reacts with $O_2^{\cdot -}$; the Cyt C is then oxidized to/from the electrode, generating a current proportional to the $O_2^{\cdot -}$ concentration [46, 75, 76]. A general schematic of the immobilization procedure using self-

assembled layers of mixed thiols is shown in **Figure 2.** Since biosensors utilize biomolecules for recognition they tend to be more selective then chemical sensors. Moreover, because they require immobilization of the biomolecule, the long-term stability of these probes might pose challenges for translation and field implementation. **Table 2** provide examples of some common microelectrode platforms for measuring ROS/RNS in biological systems. Performance characteristics for representative sensors and examples of applications are discussed in the next sections.

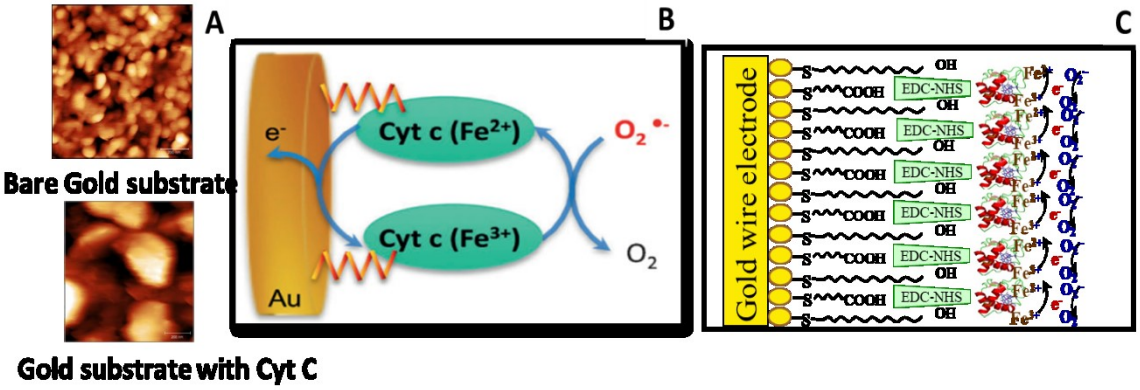


Figure 2. Electrode design and immobilization of a redox protein (Cyt c) showing: (A) changes in gold morphology before/after electrode modification studied by conductive AFM, (B), redox wiring and electrode transfer process and, (C) immobilization of Cyt c using a layer of mixed thiols on a gold wire electrode modified with Au nanoparticles.

Table 2. Examples of electrochemical sensors and biosensors and electrode designs for ROS/RNS detection in biological conditions (reproduced with permission from Ref [24]).

#	ROS/ RNS	Electrochemical Technique	Electrode materials	Working electrode	LOD	Biological system	Ref
1	H_2O_2	Fast Scan Cyclic Voltammetry	1,3-phenylenediamine	CFME	20 µM*	Rat brain	[77]
2	H_2O_2	Chronoamperometry	Pt-Pd bimetallic nanocoral	CFME	0.42 µM	A549 living cells, milk	[78]
3	H_2O_2	Amperometry, CV	Hemoglobin, SWCNT	CFME	0.23 µM	HePG2 cancer cells	[79]
4	H_2O_2	Amperometry	Au-Pd alloy NPs, Graphene Quantum Dots	CFME	500 nM	Clinical breast cancer tissue	[80]
5	H_2O_2	Amperometry	Pt NPs, Nafion, PPD	CFME	0.53 µM	*In vitro*	[81]
6	H_2O_2	Amperometry	Platinized silica nanoporous membranes	CFME Or ITO	0.01mM	Rat brain	[82]
7	H_2O_2,	Chronoamperometry	Heat treatment to create nanopores to improve catalytic performance	Heat-treated CFME	1 µM	*In vitro*	[83]
8	H_2O_2	Chronoamperometry	Core-shell 2D VS_2,@VC@N-doped carbon sheets decorated by Pd NPs	CFME	50 nM	MCS-7 cancer cells, and breast cancer tissue	[84]
9	H_2O_2	Chronoamperometry	Pt–Pd NPs, graphene oxide	CFME	0.3 µM	Raw 264.7 cells secretion	[85]
10	H_2O_2	Chronoamperometry	Au-Ag bimetallic NPs / polydopamine	CFME	0.12 µM	HepG2 cells	[86]
11	HClO/ ClO^-	DPV	Graphene Oxide, carbon nanotubes, MBS	CFME	0.5 µM	Body fluids	[87]
12	$O_2^{\cdot-}$	Chronoamperometry	MWCNTs,Ionic Liquid-Br, SOD, Prussian Blue NPs	CFME	0.42 µM	Alzheimer rat brains	[88]
13	$O_2^{\cdot-}$	DPV with ratiometric signal output	Diphenylphosphonate-2-naphthol ester, methylene blue SWCNTs	CFME	2 µM	Rat brain	[89]
14	NO	DPV	NiTSPc/nafion	CFME	0.34 µM	Zebrafish intestine	[21]

MWCNTs-Multi Walled Carbon Nanotubes, CV- Cyclic Voltammetry, BBY- Bismarck Brown Y, rGO- Reduced Graphene Oxide, FTO- Fluorine doped Tin Oxide, AgNPs- Silver Nanoparticles, CNT- Carbon Nano Tubes, DNA- Deoxyribose Nucleic Acid, APTES- (3-aminopropyl) triethoxysilane , Cyt C – Cytochrome C,Poly(5A1N)- Electropolymerized 5-amino-1-naphthol , XG- Xero Gel, PSS-Polystyrene Sulfonic Acid, BA- 5-(1,2-dithiolan-3-yl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)pent-anamide, CFME- Carbon Fiber Micro Electrode, AuNP- Gold Nanoparticles, PDDA- Poly(Diallyl Dimethil Ammonium Chloride), D-cell - Plastic disposable Carbon based Electrochemical Cell, a-NSGF- Taurine-functionalized Graphene foam, ITO-PET- Indium Tin Oxide supported on Poly-Ethylene-Terephthalate foil, CTS-Chitosan, MPNS-Microporous Polymeric Nanospheres, HTCMP-Hollow Tubular Conjugated Organic Microporous Polymer

3.2. Electrochemical sensors for H_2O_2 and superoxide radicals

H_2O_2 and superoxide $O_2^{\cdot}$ radical species are characterized by short half-life in the extra- and intracellular space and are present at low and variable concentrations due to the complex and dynamic oxidant/antioxidant system. Another radical species, $HO^{\cdot}$ has the lowest life time among the ROS and its formation is limited to the use of the Fenton reaction. Methods for the detection of $HO^{\cdot}$ are typically based on its strong oxidation properties after exposure to $HO^{\cdot}$ during the Fenton reaction [90], or by indirectly measuring the oxidation properties of $HO^{\cdot}$ towards DNA strands [91], quantified by electrochemistry using methylene blue as a mediator.

Among ROS, H_2O_2 is the easiest to detect electrochemically [19]. A CFME modified with a synthetic receptor with affinity towards H_2O_2 enabled H_2O_2 determination in a single drop of blood using differential pulse voltammetry (DPV) in the potential range -0.5 to - 0.6 V *vs* Ag/AgCl, and a linearity range of 0.5 -400 □M [92]. A carbon-based electrochemical electrode modified with Ag nanoparticles and δ-FeOOH enabled H_2O_2 detection in fetal bovine serum [93]. Electrodes functionalized with chemical mediators such as Prussian blue (PB) are a preferred modality for ROS, especially H_2O_2 detection at concentration levels down to 1.3 □M [94]. Strategies to increase selectivity of CFMEs include modification with catalytic materials like Pt, often combined with Au, Ag or carbon nanostructures for enhancing performance. A CFME functionalized with VS_2@VC@N-doped carbon sheets and Pd nanoparticles facilitated detection of H_2O_2 at -0.05 V with no major interferences from electroactive species [87].

Detection of $O_2^{\cdot}$ is complicated by the fact that, in aqueous solutions, superoxide undergoes disproportionation to H_2O_2. The disproportionation reaction is a very fast second order with respect to $O_2^{\cdot}$ ($k = 2 \times 10^5$ M^{-1} s^{-1} at 25 °C and pH of 7.4 [95]), and thus measurements are not an easy task. The standard redox potential of the $O_2/O_2^{\cdot -}$ redox couple is between 330 - 140 mV vs NHE. $O_2^{\cdot}$ detection can be achieved by direct electrochemistry using platinized CFME, or MEs modified with N-doped carbon AgNPs [96] or porous carbon [97]. A common approach to improve selectivity is to immobilize Cyt C or superoxide dismutase onto a functionalized MEs [19], or use enzyme mimetic materials such as $MnTiO_3$ microdiscs, [98] manganese phosphate [99] or nanostructured Mxenes [100] and graphene/AgNP/CeO_2/TiO_2 [101] as alternative to natural enzymes. Cyt c-based nanoporous

gold electrodes with a detection limit of 1.9 nM and a sensitivity of 1.9 nM.nM^{-1}cm^{-2} enabled the detection of $O_2^{\cdot-}$ in skeletal muscle tissue [102]. **Figure 3** shows an example of Cyt c-based electrochemical biosensor fabricated on a Au ME wire for $O_2^{\cdot-}$ detection, the electrode processes and detection mechanism. The sensitivity of the biosensor was 42.4 nA/µM and the LOD was 2.4 nM. The biosensor was tested in human blood platelets and skeletal rat limb muscles following ischemia reperfusion injury (IRI), confirming the potential of these measurements in complex biological systems [46]. A similar configuration also demonstrated performance for real time measurements of $O_2^{\cdot-}$ in the intestine [103] (**Figure 4**) .

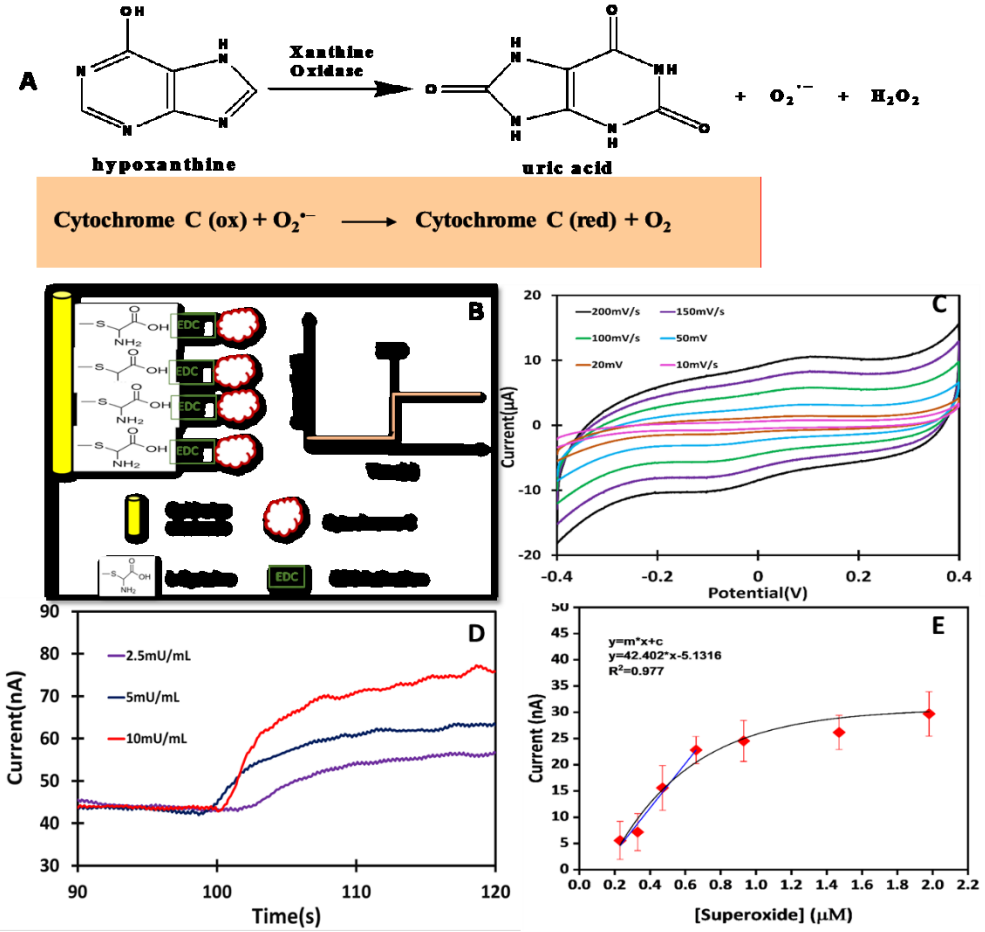


Figure 3. Example of electrochemical biosensors for superoxide used using a Cyt c modified electrode showing: A) formation of $O_2^{\cdot-}$ using hypoxanthine/xanthine oxidase, B) immobilization process using L-cysteine and EDC chemistry. C) effect of scan rate indicating a surface confined process, D) effect of different concentrations of enzymatically generated and $O_2^{\cdot-}$ obtained from different XOD levels, E) calibration curve. Reproduced with permission (Ref [46]).

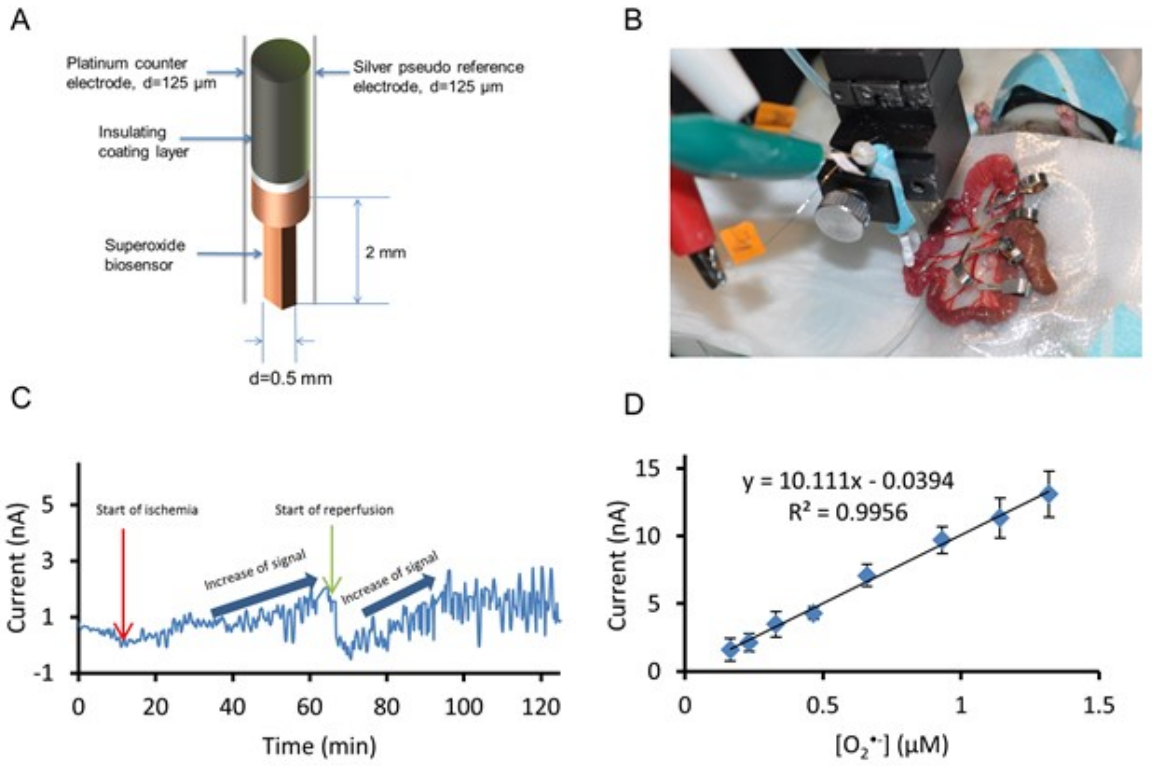


Figure 4. Example of electrochemical $O_2^{\cdot-}$ recordings using a Cyt-c thiol modified gold wire electrode implanted in the intestine. Reproduced with permission from (Ref [103]).

3.2 Electrochemical measurements of nitric oxide (NO) and peroxynitrite ($ONOO^-$)

NO is a highly reactive diffusible species, which can combine with $O_2^{\cdot-}$ to form the more toxic peroxynitrite. Electrochemical detection of NO can be accomplished based on the electrooxidation of NO, which takes place at a potential >0.8V vs Ag/AgCl using working electrodes modified with catalytic and selective membranes. Different configurations of NO sensors have been summarized is an extensive review [104]. CFMEs functionalized with Nafion [21], o-phenylene diamine (o-PD) or chitosan [21, 72] have been reported that showed high selectivity and sensitivity. I*n vivo* measurements can be accomplished with platinized or platinum wire electrodes. An electrode consisting of a glass nanopore platinized platinum electrode enabled in-depth NO profiling in the cortical and hippocampal brain areas of a rodent model [105]. Other works reported multisensing configurations for $NO^{\cdot}$ and oxygen [106], carbon monoxide [107] or potassium ions [108], which enabled real time monitoring of dynamic concentration changes and signalling of these species. A Pt disk electrode modified

with an electropolymerized permselective 5-amino-1-naphtol (Poly(5A1N)) and fluorinated xerogel enabled measurement of NO in pro-inflammatory macrophages [109], achieving a detection limit (LOD) of 1 nM and a linearity range (LR) from 0.01 to 10 □M. An electrode coated with reduced graphene oxide and platinum nanoparticles enabled NO detection in human serum, with a LOD of 52 nM and a LR from 0.25 to 40 □M [110]. A microsensor with Poly(eugenol) coating enabled high selectivity detection in the presence of H_2O_2 [111]. Other sensors integrate a flexible implantable microelectrode coated with polylactic acid and poly(trimethylene carbonate), an ultrathin Au membrane, and a poly(eugenol) film, with wireless monitoring to track NO release over a period of 5 day in the heart and joint cavity of rabbits [112]. The sensor had a LOD of 0.97 nm and a LR of 0.01-100 □M. Our group developed CFMEs modified with selective membranes to track the evolution of NO in different locations of the intestine of zebrafish embryos [21, 113]. The sensor was used as a measurement tool to evaluate changes in NO levels in response to drugs and to assess oxidative stress response as a result of nanoparticle exposure. Examples of electrochemical recordings in the zebrafish intestine, location of the electrode during implantation and Scanning Electron Microscope (SEM) image of the microelectrode before and after electrochemical treatment to increase sensitivity are shown in **Figure 5**. Measurements showed good correlation with results obtained by fluorescence imaging using the 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) dye [22].

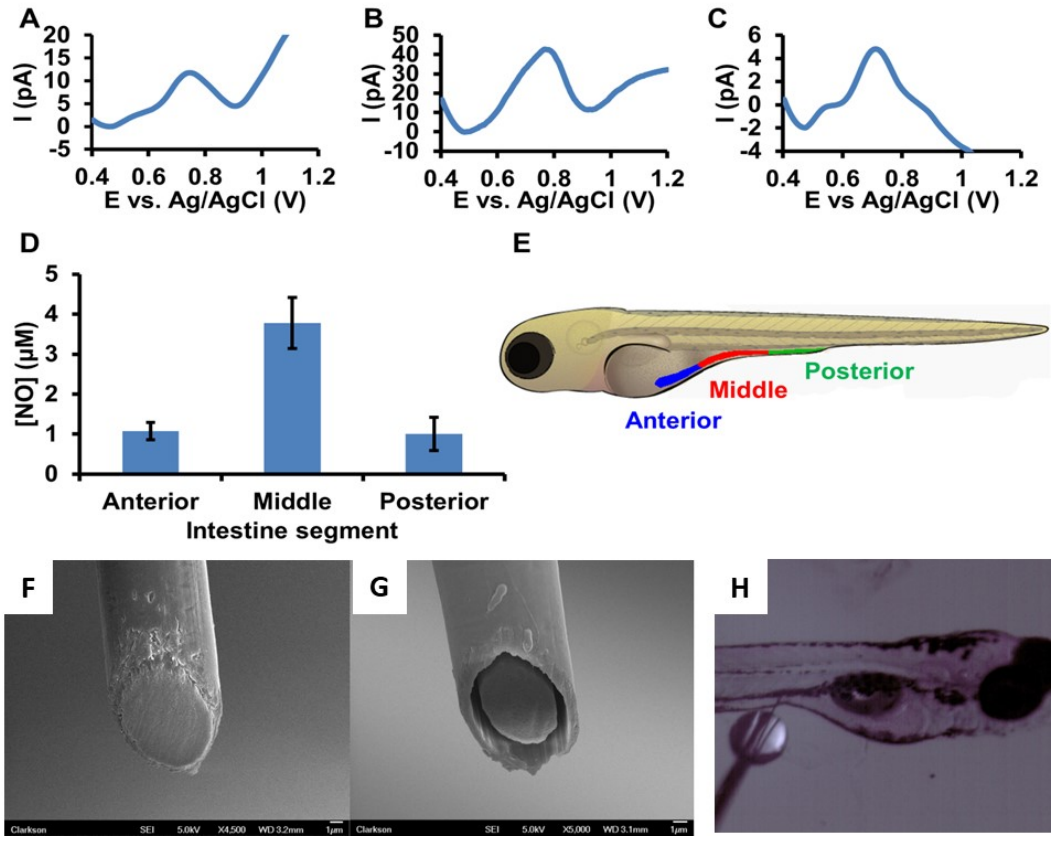


Figure 5. *In vivo* DPV recordings with the microelectrode inserted in the intestine of 5 dpf zebrafish embryos in the: anterior (A), middle (B) and posterior (C) segments. Due to the measurement being made in live embryos without the use of anesthetics, small shoulders can be seen in the voltammograms recorded *in vivo* which may be due to the movements of the embryo. NO concentrations measured *in vivo* along the intestine of 5 dpf zebrafish embryos; error bars represent standard deviations for n=3 measurements in different embryos using individual microelectrodes (D). Schematic representation of the three regions of the zebrafish embryo intestine where the sensor was implanted (E). The length of the embryo at 5 dpf is approximately 4 mm. (F, G) Scanning electron micrograph images of the carbon fiber microelectrode, before (F) and after (G) modification with NiTSPc and Nafion. Electrochemical deposition of NiTSPc takes place with etching of the carbon fiber microelectrode tip. The scale bar is 1 µm. (Figs 1, 2, with permission from Ref [49]).

Peroxynitrite ($ONOO^-$) the product formed in the fast reaction of $O_2^{\cdot}$ with NO, is relatively newer ROS/RNS species important because of its high reactivity and damaging effects. Because of its extremely short time $ONOO^-$ has been difficult to measure [114]. Measurements are further complicated by the myriad of cellular reaction and the dependence of pH and cellular environments. The $ONOO^-/ONOO^{\cdot}$ Formal potential is 0.27 V vs SSCE [115], which makes possible direct electrochemical sensing of this species. Most commonly

used electrodes are based on platinized MEs modified with electrocatalysts such as conjugated Mn complexes (e.g., tetraaminophthalocyanine manganese (II)[116], MnO_2-Hemin [117] and PEDOT-Hemin [118] layers, and polymeric nanospheres [119]). Simultaneous detection of ROS/RNS is of great interest due to inter-relation between these species, changing concentrations dynamically over time and their high reactivity. An example of multi-ROS/RNS microfluidic-based detection has been reported for ROS/RNS released by cells [120] and ratiometic measurements [121], shown in **Figure 6**.

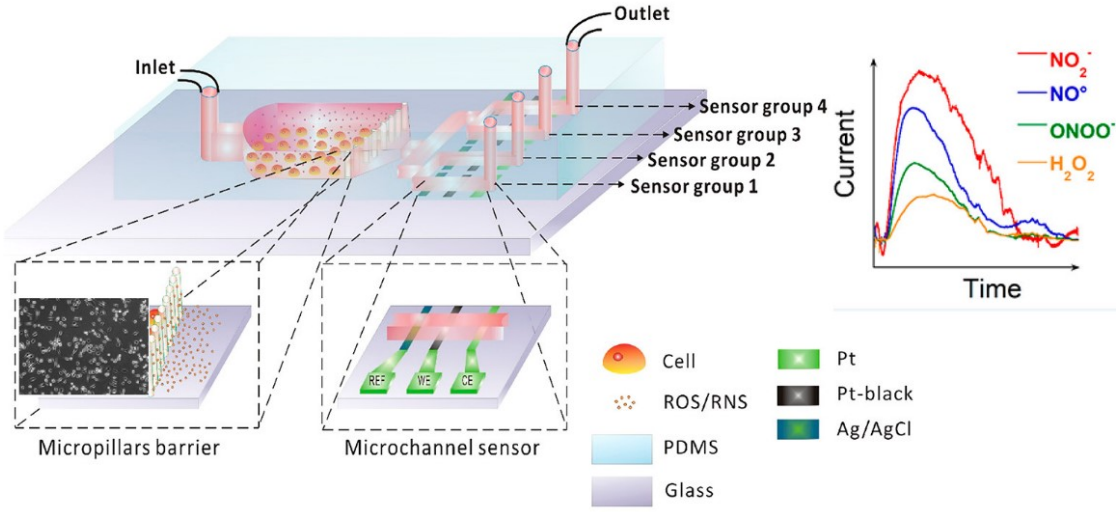


Figure 6. Multi-detection of H_2O_2, $ONOO^-$, $NO^{\cdot}$, and NO_2^- on parallel microfluidic channels (with permission from Ref [120]).

The development of electrochemical sensors for $ONOO^-$ detection is a big challenge due to its instability and short life time, as well as challenges related to the preparation and handling of $ONOO^-$ *in vitro*. Similarly to $O_2^{\cdot-}$, the validation of $ONOO^-$ sensors is often questionable. Because of a lack of selective catalysts for its detection, only a few publications report electrode modifications for $ONOO^-$ sensing, and the existent work is based exclusively on electrochemical mediators. Hemin, an iron containing porphyrin, was shown to possess electrocatalytic activity for $ONOO^-$ [122]. The sensing performance of hemin towards $ONOO^-$ was improved by synthesis of composite materials with graphene [123] or PEDOT [118]. The hemin-PEDOT material is particularly attractive for use in biological studies since its deposition on carbon fiber electrodes was already reported [124]. Cobalt-containing mediators have also been used for $ONOO^-$ detection. The performance of a cobalt porphyrin-graphene

composite material has been demonstrated for simultaneous detection of $ONOO^-$ and H_2O_2 [125]. Catalytic activity for $ONOO^-$ detection was also reported for a cobalt center-containing complex, cyanocobalamin [126]. Its use is also advantageous due to possibility of forming thin films at the electrode surface by electrochemical polymerization from monomer solutions.

4. Biological applications and translational aspects

Accurate ROS/RNS measurements are important to understand the relationship between oxidative stress and disease and even monitoring single cell events [127]. Because oxidative stress is implicated in the progression of many conditions, such measurements can be used to provide insights into the development of diseases, evaluate effectiveness of therapies and quantify ROS at subcellular levels such as for example in fibroblast and cancer cell lines [127], or in single cells to evaluate effect of treatment with chemotherapeutics [128]. The translational utility of these sensors was evaluated in biological fluids like human platelets [46] and several animal models, e.g. ROS/RNS during intestinal ischemia-reperfusion injury [103].

Despite the desirable characteristics of these sensors, several challenges remain before they can be translated to clinical work. Limitations include: 1) the risk of biofouling, or adsorption of biomolecules onto the probe, reducing detection capability, particularly for probes used in vivo [129], 2) interference from co-existing electroactive compounds [130], 3) quantitative data interpretation, 4) selectivity, 5) calibration drifts and accuracy of measurements. In particular, ROS/RNS concentrations are harder to estimate due to limited stability and high reactivity of these species. Biological media are complex and difficult to replicate for external calibration purpose, or calibration is unreliable due to lack of stability of reactive species. For these reasons, validation using alternative methods should be conducted side-by-side with calibration and during measurements in biological models [113].

Current development status of electrochemical sensors for ROS/RNS measurements demonstrates the potential of this technology for investigating complex biochemical mechanisms in biology and medicine. Their capabilities enable rapid, sensitive and real-time detection of reactive species in live organisms, with potential to provide detailed investigation of biochemical mechanisms at organ levels when implanted, and at fundamental level, in cell

populations or at single cells. These studies can elucidate biological processes and study mechanisms of disease development or injury states. The potential of these methods has been demonstrated in cells and biological media and future work can further advance this technology for broad adoption by the biological, clinical and biomedical communities. Work demonstrating intracellular measurements is also expected and can make significant contributions to the field. Development of methodologies for simultaneous detection of multiple ROS/RNS is underway examples of measurements in different biological models can decipher complex oxidative stress mechanisms and processes. The applicability of this approach for measurements in living organisms has been demonstrated and recent works provide evidence highlighting the potential of this methodology in human subjects. Such studies could provide significant information on the biochemistry of living organisms and advance clinical measurements and disease diagnostics.

5. Conclusions and trends

ROS/RNS measurements are important and because oxidative stress is implicated in the progression of many pathological conditions, ROS/RNS sensors can be used to understand the relationship between oxidative stress and disease. When carefully designed, electrochemical sensors and biosensors can monitor the appearance, evolution and continuous monitoring of ROS/RNS species, and the study of the dynamics of reactions and processes involving ROS/RNS species. However, electrochemical sensors are relatively seldom used to investigate processes in whole organisms. Their capabilities and potential adoption as biomedical tools in the clinical field is not well known by the biological and clinical communities and as a consequence, their reliability for measurements in live organisms and clinical applications is still to be proved.

Although sensors for ROS/RNS are well-developed, most work was done is standard solutions or synthetically generated species. While electrochemical sensors for ROS/RNS have demonstrated improved capabilities in recent years, their implementation requires further refinement to improve robustness, selectivity, sensitivity and reduce fouling. Improving the selectivity toward individual radicals, or developing multianalyte sensors for simultaneous quantification of radicals could increase capabilities and advance applications.

Manufacturing more MEs using advanced manufacturing methods is also needed to accelerate adoption. The developmental work paved the way in designing future clinical-ready probes for detection of ROS/RNS *in vivo* and enable monitoring physiological and pathological events in conditions such as cancer, traumatic brain injury, hypovolemic shock, and many other clinical applications.

6. References

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